

**BONE HEALING: THE EFFECT OF IMPLANTING A
HETEROGENOUS COLLAGEN PASTE**

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PREFACE

The work for this thesis was undertaken at the Department of Oral and Maxillofacial Surgery, University of Edinburgh and the Edinburgh Dental Hospital and School from 1982 to 1986. The animal work was performed at the Medical Faculty Animal Area and laboratory facilities were provided by the Department of Oral and Maxillofacial Surgery and Department of Oral Medicine and Oral Pathology.

This research includes studies which have been modified to compare the performance of the test material with previously published work; original observations are made of the conflicting results.

The clinical work and review of patients was performed personally. The radiographs were all taken by Mr. A.R. Bradshaw in the Department of Radiology, Edinburgh Dental Hospital and School. The operative surgery on animals was undertaken personally and the tissue microtomy was performed by the technical staff of the Department of Oral and Maxillofacial Surgery. The tissue culture was performed by Miss Y. Barlow who also demonstrated the ELISA technique for the author to perform jointly.

The assistance of many individuals was required to treat the patients participating in the clinical trial, to encourage their return at the appropriate times and to continually remind the defaulters. Without the assistance and co-operation of staff and patients, this work would not have been possible.

ABSTRACT

The use of autografts and allografts to replace areas of bone loss has found limited success. An ideal replacement for large areas of bone loss is a vascularised bone graft; this may prove impractical. An ideal allograft material has not been found; all materials investigated so far have shown many disadvantages. Non-biological materials may, at best, become encapsulated; inorganic and organic biological preparations may result in poor tissue healing, may induce an inflammatory immune response or be eliminated. The properties of an ideal implant material are listed.

Bone consists of an organic matrix and inorganic salts as the non-cellular elements and bone salts, in varying forms, have long been used to act as biological space fillers. The current literature suggests that collagen, which forms the major protein mass in the bone, as a matrix of heterologous origin, is both biocompatible and biodegradable and may also possess calcification or ossification properties. It may also act as a biological space maintainer and allow induction of and replacement of the area with host bone. Opinion is divided with regard to the role of collagen in this context.

A collagen preparation, specially formulated as a bone dressing and found clinically to have suitable properties has been the subject of investigation both in vivo and in vitro to determine its effect on the healing of bony cavities.

Biocompatibility is shown by its effect on human fibroblasts in tissue culture. The production of antibodies has been investigated with an enzyme-linked immunosorbant assay (ELISA) and has shown that heterologous collagen is an immunogen and produces a specific antibody response. Bony cavities in the mandible of an animal model have been used to show the effect of the implanted collagen matrix on healing. The material acts as a very effective haemostatic agent but although the healing process is comparable with a control area, the implant retards the process and does not show the uniform mode of bony replacement seen in the control area. Several reasons are suggested for the difference. Healing in the animal model is also shown radiographically and compared with a human implantation study when the material is placed in pathological bony cavities in either jaw. Similarly the human study shows that healing will take place but is retarded in the implant group.

Reports have shown that when collagen has been placed subdermally a small proportion of patients developed chronic granulomas. These findings have not been verified by this study: no untoward reactions due to the implant were shown in any part of this investigation.

Although the implant is both biocompatible and biodegradable, it is questionable whether collagen has any osteoinductive properties in its own right.

It is hoped that similar methods to those used in this work will be employed to test any new surgical materials for replacement of lost tissue.

GLOSSARY OF ABBREVIATIONS

Å	Angström units 10^{-10}
ATP	Adenosine triphosphate
B lymphocyte	Bursa equivalent lymphocyte
°C	Degrees Celsius
cm	Centimetre
EDTA	Disodium ethylene diamine tetracetate
ELISA	Enzyme Linked Immunosorbant Assay
EM	Electron microscope/microscopy
FFD	Fixed focal distance
Glasgow's MEM	Glasgow's minimal essential medium
KCl	Potassium chloride
Kg	Kilogram
KH_2PO_4	Monobasic potassium phosphate
KV	Kilovolt
(L)	Left
mg	Milligram
MgCl_2	Magnesium chloride
ml	Millilitre 10^{-3}
mm	Millimetre 10^{-3}
M solution	Molar solution
μ	Micron 10^{-6}
μl	Microlitre 10^{-6}
NaHCO_3	Sodium bicarbonate
Na_2CO_3	Sodium carbonate
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	Dibasic sodium phosphate
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	Monobasic sodium phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
nm	Nanometre 10^{-9}
PBS	Phosphate buffered saline
PBS tween	Phosphate buffered saline-Tween 20 mixture
(R)	Right
rpm	Revolutions per minute
T lymphocyte	Thymus dependent lymphocyte
ZnCl_2	Zinc chloride

GLOSSARY OF TRADE MARKS

Absele Absorbable Bone Sealant

Solubilised bovine collagen 17.5%: Stabilised bovine fibrin
17.5%: Dextran 70 8%: Glycol 30%: Water to 100%.
Ethicon Limited, P.O. Box 408, Bankhead Avenue, Edinburgh.

Achromycin

Tetracycline hydrochloride.
Lederle Laboratories, Fareham Road, Gosport, Hants.

Araldite Resin

Agar Aids, 66A Cambridge Road, Stanstead, Essex.

Avitene Microfibrillar Collagen

Avicon Inc., Fort Worth, Texas, U.S.A.

Dexon Sutures

Synthetic absorbable polyglycolic acid sutures.
Davis & Geek, Fareham Road, Gosport, Hants.

Dextran 70

Pharmacia Limited, Midsummer Boulevard, Milton Keynes.

Hypaque

Sodium diatrizoate.
Sterling-Winthrop, Onslow Street, Guildford, Surrey.

Hypnorm

Fentanyl citrate with Fluanisone.
Janssen Pharmaceuticals Limited, Grove, Wantage, Oxfordshire.

Nembutal

Pentobarbitrate Sodium.

Abbott Laboratories Limited, Queenborough, Kent.

Valium

Diazepam.

Roche Products Limited, P.O. Box 8, Welwyn Garden City, Herts.

Zenoderm Corium Implant

Porcine dermal collagen

Ethicon Limited, P.O. Box 408, Bankhead Avenue, Edinburgh.

Zyderm Collagen Implant

Bovine collagen dispensed in PBS with lignocaine.

Collagen Corporation, 2455 Faber Place, Palo Alto, California, U.S.A.

INTRODUCTION

When tissue has been lost, the aim of a surgeon is to restore the structure and integrity of that tissue wherever possible. When normal form and function cannot be restored, the body will replace the defect with fibrous tissue and such healing will often result in a marked loss of tissue contour. Surgeons have tried to restore aesthetic and functional defects with autograft and allograft materials. The use of alloplastic materials to replace lost tissue has frequently been unrewarding for patient and surgeon alike. New materials are met with much enthusiasm initially, only later to be condemned, as rejection and chronic inflammation ensue, with subsequent tissue necrosis and fibrosis indicating that such materials lacked biocompatibility.

Heterogenous materials have been used to replace areas of bone which have been destroyed by disease. Osteomyelitic cavities were common in the pre-antibiotic era and as early as 1889 decalcified bovine cortical bone chips were used to fill such cavities (Oikarinen & Korhonen 1979). Between the 1920's and 1960's plaster of Paris was a popular material to fill bone cavities (Nystrom 1928; Nielson 1944; Bahn 1966). This material was partially successful being resorbed concentrically and gradually replaced with ossifying granulation tissue.

Hydroxyapatite forms a major portion of the inorganic salts in bone and in various forms is used to fill bony defects and augment lost areas of bone. The material is biocompatible but not biodegradable (TaHERI et al 1972). Certain corals appear to be similar in structure to dried bone and it was hoped that these

materials alone, or in conjunction with hydroxyapatite would be better suited for bone replacement (Holmes 1979). Limited success has been achieved. More recently much enthusiasm has been shown in the use of ceramic materials to replace bone and joints (Driskell et al 1972, 1973; Davis et al 1972). Long term results of these materials is awaited.

The use of a wide variety of materials indicates an ideal alloplastic material has yet to be found. Such materials listed above could at best be described as "minimally reactive", in other words if placed within a biological system they would excite a non-specific inflammatory or foreign body reaction of a chronic nature and probably become walled off by a thin fibrous capsule (Herch 1980). Until recently, little attention was given to the other non-cellular organic component of bone, its collagen matrix. It is known that collagen in connective tissues consists of rigid triple helical molecules that provide structural integrity by virtue of their staggered arrangement which depends on the covalent intermolecular and intramolecular cross linkages (Nimmi 1973). When the cross linking is increased by aldehydes or other methods, they are said to excite a much reduced inflammatory reaction (Defalco 1970). Collagen can now be produced in a highly purified "pure" form and such a heterologous collagen preparation could act as both a biocompatible and biodegradable replacement scaffold in bony defects. When collagen cross linking is increased, the rate of breakdown of the material is prolonged so it is conceivable that if the material remains in situ while the area is calcified or gradually replaced by bone, full restoration of the normal bony contour could be achieved.

The advantage of collagen over the inorganic allografts would be complete degradation of the implant. If collagen is to be placed in bony cavities it must remain in situ while the area is healing to provide the correct bulk and contour for complete tissue restoration.

Several collagen preparations are available commercially. Zyderm™ is a soluble collagen preparation used to replace lost subcutaneous tissue. It is injected as a solution and would therefore be unsuitable for bony implantation. Avitene™ is prepared as a collagen 'powdered fleece' in small dry particles which easily adhere to a bleeding surface. It is used as a haemostatic agent (Hiatt et al 1973) but is again not physically suitable for filling bony cavities. A soluble collagen-fibrin combination produced as a "putty" consistency was produced by Ethicon Ltd.¹ as Absele™. Such a material could have physical properties better suited to bony implantation. Part of the preceding work for this thesis involved the use of this material and its assessment clinically as a bony dressing for a dry socket, a common sequel to about 4% of all teeth extracted.

Dry socket, a term that was first used in the literature in 1896 (Crawford 1896) is believed to be the result of early or excessive fibrinolysis of the blood clot in the post-extraction socket (Birch 1973). The clot is fragmented and lost, leaving an area of exposed alveolar bone. Pain and delayed healing result. Many materials have been used to seal the exposed bone and act as an antiseptic or obtundant. Some are said to have beneficial

¹ Ethicon Ltd., Bankhead Avenue, Edinburgh, Scotland.

effects in pain relief but all may have adverse effects on healing. The author felt a more effective treatment may be provided by a resorbable biological matrix and as collagen has been shown to be compatible with human tissues, it could have a positive effect on bone healing.

A small clinical study was undertaken using Absele™ but the material proved unsuitable on many counts. It was felt that the fibrin content may have caused an increased inflammatory reaction and the material itself was too soluble in oral fluids.

From the experience gained and in consultation with the manufacturer, it was felt that in theory a combination of the soluble collagen component of Absele™ and an insoluble collagen material would provide a more robust material that would remain in the wound for a longer period and thus allow granulation tissue to grow into the area. The soluble portion should be more easily phagocytosed and replaced by osteogenic granulation tissue, whilst the insoluble portion would remain as a matrix to allow healing to progress throughout the defect before it was removed. It was felt that the presence of fibrin would not be of benefit and it was therefore omitted.

Soluble and insoluble collagen were prepared from bovine corium after removal of hair and epidermis. The skin was ground with acetic acid at 4°C to prevent heat denaturation of the triple helix. The resultant material was protease digested to remove the telopeptides and non-collagenous proteins. The acidic salt was filtered, centrifuged, refiltered and dialysed against a phosphate buffer before precipitation at 37°C. Insoluble collagen was prepared as a water insoluble, glutaraldehyde cross linked acid

salt of natural collagen. Details of the method of production are retained by the manufacturer.

Varying mixtures of these soluble and insoluble collagens were prepared and supplied as powders or mixed in varying proportions with isotonic saline and a small amount of a tackifying agent (Dextran 70TM). The samples were mixed to a semi-solid consistency which could be extruded from a large orifice syringe. The varying compounds were packaged in sterile syringes in plastic envelopes and sterilised by cobalt irradiation. Initially eight variants of the formula differing in the proportions of soluble and insoluble collagens were prepared.

Each material was assessed clinically using the following criteria. That they should :-

- i) have no irritant effect on the surrounding tissues,
- ii) be incorporated or absorbed,
- iii) act as a bone sealant to oral fluids,
- iv) be stable in serum with no volume change,
- v) be non-antigenic,
- vi) be easy to apply, and
- vii) improve healing.

Little or no irritant effects or adverse tissue reactions were noted with any of the test materials but the more soluble materials were easily washed away and the more insoluble the material, the more difficult it was to handle. A narrow range of suitable materials was obtained and with minor modification, a suitable formula was finally devised.

The collagen paste (Fig. 1) was supplied by the manufacturer in a standard 1 ml plastic syringe inside a sealed clear envelope,

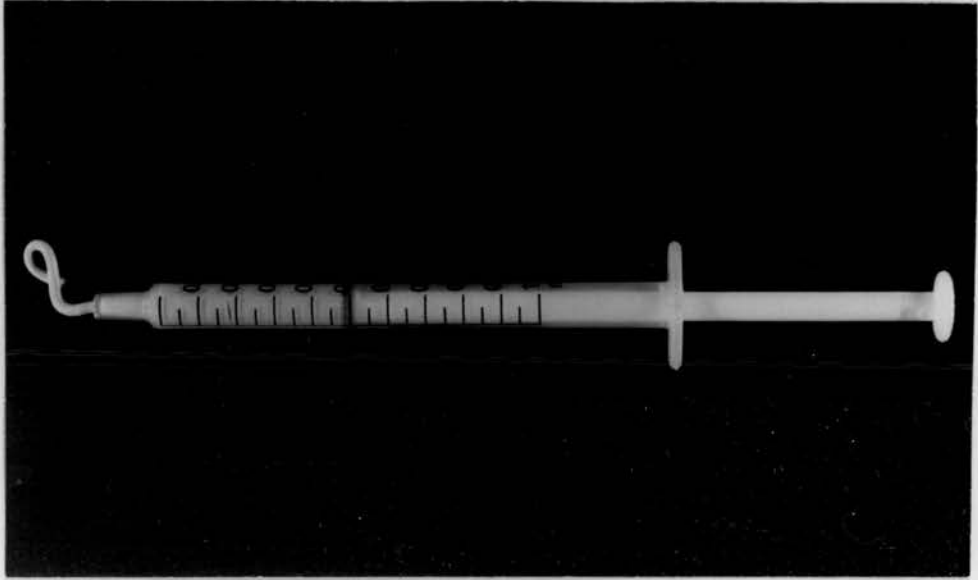


Fig. 1 Collagen implant material in sterile syringe ready for insertion into bony cavity.

the completed package being sterilised by cobalt irradiation.

The formula of the test material used is as follows:-

Soluble collagen	22%
Insoluble collagen	22%
Dextran 70™	8%
Isotonic saline	48%

The material has been patented for use in bone healing (British Patent No. 8234504).

This material was then used in a clinical trial comparing it with a standard material (Zinc oxide/oil of cloves) for the treatment of a dry socket as a dressing material only. (Permission was sought and given by the Ethical Committee for this work.)

The results were encouraging and it was felt that sufficient material remained in the socket to create a suitable environment for healing by secondary granulation to begin. The surrounding tissues appeared healthy at all times. The ultimate fate of the dressing was unclear. Concomitantly with this clinical work, implantation of this material in the laboratory rat was begun using a 2 mm diameter, 4 mm depth of hole in the mandible. No real conclusions were drawn from this laboratory work as it was found that although the areas were clearly marked with sutures, the defect healed so quickly it proved difficult to relocate and little information could therefore be gained.

However, the clinical study showed that the test material appeared to fulfil most of the criteria set out for an ideal bone dressing and healing was improved with the collagen dressed areas compared with those dressed with the previously accepted standard dressing (Mitchell 1986).

The clinical work showed promise and it appeared to the author that this collagen preparation, if proven to be biocompatible and biodegradable, could have a positive beneficial effect on the healing of bony defects with complete restoration of tissue integrity and contour.

The following study was thus designed to evaluate wound healing and the inflammatory response to this material in vivo and in vitro together with the immunological response produced by its use.

CHAPTER 1

A REVIEW OF THE LITERATURE PERTAINING TO BONE
HEALING AND THE USE OF BIOCOMPATIBLE MATERIALS

1. HISTORICAL CONCEPTS OF BONE

The nature of bone has been the subject of debate and controversy from earliest times. Renowned Greek philosophers regarded the tissue as an inert scaffold. Aristotle (De generatione animalum) regarded flesh to be produced of the purest materials and bone constructed from the residue. Such a view of the inorganic nature of bone was shared by Hippocrates (De carnibus) and Galen (Opera omnia) who further believed that bone was solidified by heat which was produced internally to bake and dry them (Enlow 1963).

In the 16th Century, Andreas Vesalius (1514-1564) demonstrated that bone was formed initially in cartilage (De humani corporis fabrica libri septem 1543) and Gabriele Fallopius (1523-1562) identified the epiphyseal plate. Both writers held views that would not be further elucidated for several centuries.

Until the 18th Century, views on bone constituents were varied. The anatomist Alexander Monro (1733-1817) regarded the constituents of bone as phlegm, spirit, volatile salt, fetid oil and black caput mortuum whose proportions varied with age. Caspar Bartholin (1655-1738) thought bone developed from a mucous substance which later filled with sinew, became cartilaginous and hardened into bone. Marcello Malpighi (1628-1694) also considered bone to be fibrous in nature, having a matrix of fibres and filaments with an intermediate osseous juice.

During the early 18th Century, the microscope provided a more rational approach to the structure of bone. Antoni van Leeuwenhoek (1632-1723) wrote to the Royal Society in 1693 describing his microscopic studies of bone. Examination of pieces of broken bone had shown a solid matrix penetrated by "tubuli" of varying size (Hancox 1972). Early microscopists viewed both plant and animal tissues and made direct comparisons. Tree bark was directly compared with periosteum and their internal structures thought to have similar roles. Clopton Havers (1650-1702) also identified canals in bone describing them as lying in longitudinal and transverse directions. The former he regarded as effluent channels for the expulsion of offensive vapours and the latter were to transmit medullary oil to other regions of the bone (Enlow 1963).

This view was contradicted by Jacob Winslow (1669-1760) and Bernhard Albinus (1697-1770) who both clearly confirmed an elaborate vascular supply within the canal system. John Howship (1781-1841), surveying longitudinal bone canals, showed a loss in part of their smooth lining; in some areas a rough and uneven appearance was seen with comparable areas externally exhibiting a granular appearance. This observation demonstrated bone resorption and the name "lacuna" linked with Howship was suggested by Todd & Bowerman (1845) who also introduced the concept of the "Haversian system" (Hancox 1972).

John Goodsir (1814-1867) described bone "corpuscles" and their canaliculi. He noted these cells contained nuclei and by studying young and adult bone, he showed the close relationship between the newly formed bone matrix and osteogenic cells (Goodsir reprinted 1965). His essays published in 1845 illustrate the change from descriptive osteology to a dynamic study of bone as a tissue.

Observation of bone physiology had been recorded in Greek and Roman times; the dye madder (alizarin) had been seen to stain bone. This was rediscovered in the 16th Century but the significance not appreciated. Later John Belchier (1706-1785) demonstrated that hogs fed on red madder developed stained bones with no staining in other tissues. The observation that the staining only affected growing bone marked the beginning of bone physiology (Belchier reprinted 1965). The nature of bone growth was further elucidated by John Hunter (1728-1793) who realised that bone underwent a process of resorption in parallel with osteogenesis.

The accepted principle that bone transformed directly from cartilage by hardening was repudiated by Heinrich Muller (1820-1864) who showed that cartilage was a provisional tissue which was destroyed and replaced by bone with bone forming cells originating in the marrow. The osteoclast as a cell type was described by Charles Robin (1821-1885) in 1864 and Koelliker in 1872 established the relationship between resorption, Howship's lacunae and osteoclasts. Edward Klein (1844-1925) in 1883 showed that the matrix of bone yielded gelatin on boiling and it must therefore be of a dense connective tissue type.

Woven bone was first noticed by Muller in 1860 and further fully described by Koelliker in 1886.

Thus by the end of the 19th Century, bone was known to be a calcified tissue with an organised structure, a cellular complement of different cells and an organic collagenous matrix. This improved knowledge of bone histology and physiology had therefore established the foundations on which our present concept of bone as an active tissue was built.

2. CURRENT CONCEPTS OF BONE REPAIR

A knowledge of the process of bone repair is necessary to understand the influence that an implant material may have on the host tissue.

The following brief account is a compilation of current texts on fracture healing and endochondrial ossification of the epiphysis with some current theories on areas of debate.

Repair of bone is generally by secondary intention which can be compared initially to wound healing in any wound when the lost tissue is replaced firstly with a vascular granulation tissue.

Primary bone healing is able to take place. This is demonstrated in the rat tibia where holes of up to 800 μ can be drilled and will be found to heal by primary intention. In these experiments no cartilage or connective tissue was formed, the cavity was closed by a sealing callus and bony splinters resulting from the cutting were found to be incorporated into the osseous framework (Draenert & Draenert 1980).

Secondary bone repair can be divided into several well recognised phases and each can be regarded as a series of "still photographs" in sequence. Attempts have been made to construct a continuous sequence but this obviously has limitations.

Most texts on bone healing divide the events into the following well recognised stages:-

1. Haemorrhage, coagulation, acute inflammatory reaction and necrosis.
2. Proliferation of an osteogenic repair tissue.
3. Formation of new bone, fibrous tissue and not uncommonly cartilage in the damaged area.

4. Bony defect filled with a woven bone callus.
5. Remodelling of callus.

When any tissue is damaged, blood vessels are torn and blood extravasates into the damaged area and the surrounding soft tissues. The first event is therefore blood coagulation in the defect. Platelets play an important role in the blood clotting; their haemostatic action is achieved by direct contact with exposed type III collagen fibrils in damaged blood vessels, which results in a release of platelet factors to initiate their aggregation (Wilner et al 1968). Factors released by platelet aggregation thus set off the theoretical cascade coagulation system (MacFarlane 1967) and it is now considered that such factors also have a direct effect on local tissue fibroblasts to induce proliferation in the early stages of repair (Rutherford & Ross 1976). The formed clot acts as a foreign body which must be removed and replaced.

Classically the polymorphonuclear leukocytes invade the area after blood coagulation. Their presence is obviously useful when a damaged area is infected but a direct role on the healing process is in doubt. In the neutropenic host no impairment to the healing of a non-infected wound has been found (Simpson & Ross 1972; Dale & Wolff 1971). This is in conflict with earlier reports of the function of the leukocyte in tissue repair (Page & Good 1958). The polymorph reaction becomes maximal at about 24 hours then remains or recedes depending on wound contamination. This acute inflammatory response is seen well beyond the area of bony damage and penetrates deep into any necrotic zone in the bone marrow.

After 24 hours blood monocytes are seen entering the wound reaching a peak at about 48 hours and remaining for several weeks.

They are believed to modulate into macrophages which can phagocytose coagulum debris when haemoglobin is degraded to haemosiderin; with the aid of local tissue giant cells they also remove collagen fragments and bone debris.

The cell population is now seen to increase with inclusion of lymphocytes and mast cells. The latter may keep the inflammatory response in progress by the release of agents such as histamine or 5 hydroxytryptamine; the former are capable of recognising antigen and initiating an immune response.

Within the first 24-36 hours, a new cell population and a vascular tissue are seen to be invading the damaged area. The cellular component has the facility to form bone, cartilage and fibrous tissue. These mesenchymal cells would appear to be derived from the surrounding non-injured tissue, the bone medulla and the cellular layer of the periosteum. It was originally thought that these cellular elements were derived from blood monocytes but wound healing studies using parabiotic rats, one of which had previously been irradiated, have shown that leukocytes, lymphocytes and macrophages are derived from the circulation while fibroblast derived tissue is from the local cell population (Ross et al 1970).

The musculature around the damaged bone is seen to be hyperaemic and heavily infiltrated with leukocytes and monocytes; it is from this surrounding mesodermal tissue and from the medullary cavity that the new vascular sprouts are seen to arise and supply the proliferating periosteal cells.

The fibrinolytic system is actively breaking the fibrin clot into degradation products and together with local tissue enzymes

and macrophages phagocytosing any other debris. Thus the area into which capillaries will grow is rendered more fluid and this will allow migration of the capillary arcades. These capillaries are closely supported by mesenchymal cells, probably fibroblastic in type, which can be demonstrated using an anticollagen antibody technique (Ross 1980).

This migrating tissue, termed osteogenic granulation tissue consists of three components; mesenchymal cells, able to differentiate into osteoblasts, chondroblasts or fibroblasts; other cellular elements, lymphocytes, monocytes and histiocytes, and the budding capillary loops which will later canalise to form functioning arcades. The proliferating and migrating vessels are well demonstrated with microangiographic techniques (Sevitt 1971; Carlsson et al 1960; Albrektsson 1981).

If the nutrient artery to the area is damaged, it has been shown that the response in the medulla is greatly slowed when compared with that of the periosteum (Gothman 1961).

The osteogenic granulation tissue begins as multiple isolated foci which expand and coalesce. As the foci expand, the solid vascular buds are seen to precede open capillaries and in these vascularised areas, there is early evidence of osteogenesis with deposition of calcium salts and the production of an immature bone.

Over the past 50 or so years, many theories have been put forward to explain tissue calcification. It would appear that in our present state of knowledge the evidence suggests that "crystal nucleation" is the initial mechanism allowing bone to calcify. The nucleation also seems to be dependent on cellular metabolism and the presence of a receptive matrix. Indeed, as early as 1921,

before the evidence of the electron microscope (E.M.) a theory of tissue calcification based on a specific interaction between matrix proteins and calcium ions was propounded (Freudentberg & Gyorgy 1921). The theory suggested that calcium ions could be bound to a protein and concentrated in a protein-calcium matrix which would then bind phosphate ions, thereby releasing calcium phosphate and allowing reformation of the calcium-protein complex. Although simple, this seems to have been the first molecular theory of calcification.

The use of E.M. has greatly increased the knowledge on calcification of tissues. It has shown that the first calcium salts are deposited a short distance away from the cells in an amorphous matrix between cells (Fitton-Jackson 1960). It would appear that there is a change in the matrix around the cells which precedes calcification. These observations on the E.M. correlate with the histochemical findings that where calcification was imminent the area showed metachromasia to toluidine blue. This reaction has pointed to chondroitin sulphate as an important agent in calcification (Sobel 1955) but against this, is that chondroitin sulphate is also present in the ground substance of non-calcifying hyaline cartilage.

Collagen has also been implicated as the "local agent" in tissue calcification. In vitro studies have shown that when fibrils of collagen with a $640\overset{\circ}{\text{\AA}}$ axial repeat pattern are precipitated from solution, they are able to initiate the nucleation of apatite crystals from a calcium phosphate solution (Glimcher & Krane 1968). Only collagen with the $640\overset{\circ}{\text{\AA}}$ pattern was able to do this. Electron diffraction studies on undecalcified

avian bone have identified particles of hydroxyapatite localised in a circular fashion at regular intervals along the collagen fibres (Randall 1956; Fitton-Jackson 1960). A close relationship between the banding pattern of collagen and the position of growing crystals has been shown (Bachra 1970).

The role played by alkaline phosphatase in calcification is still unclear. The enzyme appears in the intercellular matrix in developing bones and calcifying cartilage. It is easily measured and found to be increased in growing bones and when bone turnover is increased. Its high position in the theory of calcification may thus be due to the ease of demonstrating its presence.

Thus in the initial stage of calcification, the "seeding", may be achieved solely by the collagen fibrils; as a result of interaction with collagen and chondroitin sulphate or by the phosphorylation of collagen or other components of ground substance.

Against collagen solely as the seeding agent is that skin and tendon also contain $640\overset{\circ}{\text{A}}$ bonded collagen and these tissues do not calcify under normal circumstances. A major source of collagen type I with $640\overset{\circ}{\text{A}}$ is rat tail and this structure also does not calcify (Bachra & Fisher 1968). E.M. studies on calcifying cartilage and embryonic bone have also shown mucopolysaccharide containing vesicles in the extra-cellular matrix which are seen to accumulate apatite but have no direct relationship with collagen fibres (Anderson 1969). It has also been seen that a purified protein-polysaccharide material from hyaline cartilage can inhibit the precipitation of apatite from a super-saturated solution.

We are left therefore to decide whether tissue calcification is an active process, an inhibited reaction or a balance between these

depending on the local environment. Calcification does however seem to be dependent upon A.T.P. formation which suggests that mineralisation must in part be under cellular control (Cartier & Lanzetta 1961). This cellular control could explain the influence that hormones and vitamins have on mineral balance.

Whatever the true method of tissue calcification, new bone formation may be seen as early as day 7-10. At this stage a fine network of reticulin fibres is formed radiating from the osteoblasts which condense to become collagen fibrils around which calcification takes place to form osteoid tissue. Osteogenesis extends into the medulla as a vascular spearhead and new bone is laid down as seams on existing trabeculae, some of which may be necrotic; or as isolated foci in the marrow cavity. Thus by two weeks much of the damaged marrow has been invaded by granulation tissue and new spongy bone is being formed to fill the lost marrow space. The proliferating vessels form a prominent series of parallel structures directed across the gap and between them tiny vessels are seen at right angles. These vessels and cells which accompany them cross the bony deficit restoring medullary circulation and by selective growth also restore the nutrient artery. All the necrotic bone in the area is resorbed by osteoclasts and the medullary space is therefore seen to increase in capacity (Sevitt 1981).

At times islands of hyaline cartilage are formed in the fracture callus and they may be prolific in the periosteal callus. This is not the usual pattern of bone healing seen in man; ossification is nearly always in progress before cartilage is visible as the bony callus is not formed by the systematic

endochondral ossification of cartilage (Sevitt 1981). The amount of cartilage seen is variable and may be formed excessively where a large amount of callus is deposited with little or none at all being found when only small quantities of callus are produced. Cartilage is relatively avascular and seems to be formed preferentially in areas which are relatively ischaemic. Its presence may be related to a paucity of vessels in the repair tissue for when cartilage is transplanted to an extraskeletal site where there is an unimpaired blood supply, then early endochondral ossification is stimulated (Urist et al 1965). Thus cartilage may be temporarily laid down as a remaining stimulus for later ossification when conditions become more favourable.

There are differences between endochondral ossification seen in repair cartilage and at an epiphyseal plate. In callus, the sites of cartilage activity are multicentre and the ossification process is self limiting. Cartilage cells tend to be irregularly arranged in callus compared with the regular pattern of epiphyseal cartilage cells. In callus also, the ossification of cartilage is always from the periphery with vessels penetrating at a number of points. They form short tunnels in the areas of chondrolysis with subsequent formation of small bone nodules in these areas which coalesce to become woven bone.

Early work on fracture healing in rats showed a predominance of cartilage in the initial repair and it is this that led to the initial misunderstanding of bone healing in man. In man the periosteal callus is important in fracture healing in its action as the strut for bone stabilisation.

Periosteal repair arises in the inner osteogenic layer of periosteum and cellular proliferation begins some distance away from the damaged area. Cells from this area multiply and migrate, laying down new bone as they do on the outer surface of the contiguous cortex. Bridging comes from both sides of the damaged bone and is especially prolific under tissues, such as muscle, with a good blood supply (Gothman 1961). The advancing cellular periosteum lifts the fibrous layer of periosteum off the cortex and the two fronts advance to form an arch over the damaged bone which is obviously more easily achieved if the fibrous periosteum has remained intact. On the inner aspect of this arch a space, the callus space, is left. This area is initially filled with clot and debris but later infiltrated by histiocytes and the mesenchymal cells to form cartilage and bone.

At the height of callus formation, a series of slender arteries arranged in parallel to each other and passing perpendicular or oblique to the long axis of the bone, encroaches the callus space. They branch within the callus and later communicate with vessels in the cortex and in the medullary cavity. When good union occurs, they are seen to regress and disappear during the remodelling process. The initial woven bone formed does not seem to have a regular structure but rapid rearrangement of this new bone begins as soon as it is formed. Certain portions of the first trabeculae formed are resorbed by osteoclasts, while others are strengthened by osteoblasts. This action depends on the stress to which the callus is subjected. By a process of adaptation the bulky original osseous superstructure is gradually resorbed as

a firm attachment between old and new bone becomes established. This superstructure lost during the stage of remodelling, may, in man, take several months or years to be removed.

This outline of bone repair will be used to view and explain the effects of the collagen implant on the stages of healing.

3. THE USE OF BIOCOMPATIBLE MATERIALS TO REPLACE AREAS OF BONE

The use of implants in maxillofacial surgery has a long history and a wide range of materials have been evaluated. Any material used to replace bone should be stable in the presence of body fluids and produce no cytotoxic effects on neighbouring tissue. Also it is advantageous that as well as being biocompatible, the material should be biodegradable and therefore entirely replaced by the host tissue.

Bone as a tissue shows a remarkable potential for repair in the laboratory animal and in the young subject. For example, if ribs are removed for grafting and the periosteum is left in situ, new bone may be deposited at the donor site and the rib restored. Bone will heal after destruction by disease, surgery or trauma and be repaired whenever conditions are favourable, but there are limits to its healing potential. The time taken for healing may also vary. If there is a gap left between bone ends then, under favourable conditions, there will eventually be sound union, but the time for repair may be somewhat increased. The poor repair of enucleated cyst cavities in the maxilla illustrates the poor potential of this bone for complete osseous regeneration in man (MacIntyre & Speculand 1983). A similar situation is found with large cavities in the mandible of the dog, in that the healing in these areas

ceases with the formation of fibrous tissue (Hjorting-Hanson & Andreason 1971).

In maxillofacial practice there are therefore situations where the use of an implant to replace lost bony tissue is advantageous. Such implants could be used to replace areas of excised bone in the treatment of malignant disease; to increase osteogenic activity in uniting fractures: to encourage bony union in osteotomies: to restore bony contour often as onlay grafts and to fill bony cavities after excision of pathological tissue.

When large areas of bone are lost, the most advantageous method of replacement is with a vascularised bone graft, to restore contour, consistency and function. If a good vascular pedicle is provided these grafts survive well (Snyder et al 1970; Taylor et al 1975). More recently, mandibular reconstruction using free osteocutaneous flaps have been successful and these can be taken from more distant sites (MacLeod & Robinson 1982). Success depends on a viable vascular pedicle and a functional microvascular anastomosis. If the blood supply is poor these grafts can alternatively survive as fresh non-vital autogenous bone grafts still with good osteo-inductive potential. Thus vascularised and free bone grafts obviously have a major role in mandibular reconstruction after resections, but when small areas of bone loss are to be restored, they are impractical.

To induce osteogenesis in areas of poor bony healing, non-vascularised bone has been used in the form of autografts, homografts and heterologous grafts together with a variety of "bone substitute" implants such as metallic prostheses, ceramics and polymers. The following short account is an evaluation of some of

these materials which are used in clinical and experimental fields.

Autologous non-vital bone grafts usually from the iliac crest or rib areas are used extensively in maxillofacial surgery. Results are varied. In a recent report quoting 72 cases (Kruger & Krumholz 1984), 10 patients treated with a primary rib graft and 62 with a secondary rib or iliac crest graft, 20% of the primary grafts were lost compared with 3.2% of the secondary grafts. The incidence of pseudarthrosis was 30% in the primary group and 15% in the secondary. An obvious disadvantage of autogenous grafting is that a second procedure is required to harvest the bone. Although autografts clinically seem to do well when transplanted, the osteocytes do not survive (Ham 1952). Cells of the periosteum and undifferentiated marrow cells may survive, and repopulation is derived from these cells and from the marrow adjacent to the implant site (Nade 1977). Where strength is required a strut of cortical bone is advantageous, cancellous bone however does show more rapid bone replacement as revascularisation of the more dense bone is slower.

It would be simpler to replace lost bone with similar bone that was not harvested from the patient. Both homograft and heterograft bone replacements have been widely used. A stored supply of human bone from a bone bank has been used in spinal fusion (Cloward 1980). The results indicate that the percentage "take" of this gas sterilised cadaver bone equals that achieved with autogenous grafts.

Homografts and heterografts will trigger an immune response (Langer et al 1975; Muscolo et al 1976). To preserve bone grafts and to reduce their antigenicity, bone has been treated chemically

(Urist et al 1975; Goncalves & Merzel 1976) by freeze-drying (Burwell 1976), by decalcification and demineralisation (Narang et al 1971, 1972), and by lyophilisation. Cellular elements are destroyed, in particular the highly antigenic cell membranes, but other proteinous materials still remain which may be antigenic. Several investigators including Burwell et al (1963) claimed that freeze-drying bone grafts results in loss of immunogenicity, but fresh, deep frozen or lyophilised allogenic cortical and cancellous bone is still antigenically active in experimental animals as reported by several investigators (Langer et al 1975; Muscolo et al 1976; Nisbet 1977).

All allogenic grafts are largely composed of an acellular inorganic matrix which has to undergo resorption and replacement by host bone. In comparison with an autologous bone graft, homograft and heterografts show a vascular penetration which is six times less and large areas of the allografts are totally excluded from the circulation and may act as sequestrae (Stringa 1957). The success of such grafts is totally dependent on the vascularity of the recipient site (Marciani et al 1977). In the rat model, bone heterografts behave as active foreign bodies often with total loss (Goncalves & Merzel 1976). When allograft bone is used, results with such grafts are variable but said to be improved if an autologous marrow graft is used in addition, the latter providing the lost cellular elements, the former the mechanical support (Nade 1977). Decalcified and demineralised bone grafts have been used since 1898 (Oikarinen & Korhonen 1979) initially to fill bony cavities resulting from osteomyelitis. Defatted calf kiel bone has been extensively used in Europe; it is claimed to be osteoinductive

and have no immunological complications (Taheri & Gueramy 1972). The vascularisation of preserved bone allografts is said to be slower than with autogenous bone grafts because of the density of demineralised cortical bone and the graft versus host response (Oikarinen & Korhonen 1979). The osteoinductive potential of decalcified allogenic bone is said to be due to the uncovering of calcification initiator non-collagenous protein (C.I.P.) which is usually combined with bone morphogenic protein in tandem to bone collagen. The release of C.I.P. is considered to initiate calcification. Therefore the effects on osteogenesis of any form of preserved bone are variable but improved results for mandibular reconstruction are claimed with allogenic freeze-dried rib graft in combination with autogenic marrow. Good revascularisation and resorption with replacement was seen in 53 out of 56 patients in a recent study (Kline & Rimmer 1983).

Within the last decade or so, more interest has been shown in corals and ceramics which are said to have an osteoinductive potential themselves.

Porites coral, a reef building coral, has a morphology not dissimilar to dried bone, so the concept of supplying a non-vital matrix from such a source seemed attractive (Holmes 1979). The early results would suggest that the material is biocompatible and Holmes reports that 29% of his mandibular implants were replaced by lamellar bone after one year. An obvious advantage of corals is that their strength is retained throughout the healing process. Souyris et al (1985) report their implantation study of madreporaria corals into the cranium and facial bones of animals. True ossification is said to be seen with the wide porous varieties and

with the others good tolerance and stability was reported. They now report a three year study which is favourable for the use of corals to stabilise osteotomies, for bone apposition and to treat periodontal lesions. It is too early as yet to comment critically on this work but as the skeleton of the corals consists of a high proportion of calcium carbonate which can be converted to calcium apatite their behaviour may be similar to other ceramic implants (Roy & Linnehan 1974).

Ceramics in the simplest form of calcium sulphate dihydrate (plaster of Paris) were first used in 1892 and several investigators have continued using plaster as it excited minimal tissue reaction, became resorbed and replaced with new bone (Peltier 1961). Plaster has not been used extensively because of its poor mechanical strength and because its rate of resorption is greater than that for new bone formation (Frame 1980). It has been used mostly to fill bony cavities and is resorbed and replaced by granulation tissue from the walls of the cavity with partial, or, in younger patients, total recalcification (Nielson 1944). By modifying the material with a coating of cyanoacrylate, the rate of resorption has been prolonged and, it is claimed, without impairment to bony healing. However the implants failed to completely bridge circular defects in the rabbit skull (Frame 1980).

Tricalcium phosphate reacts with water to yield a substance crystallographically identical to hydroxyapatite (de Groot 1980). There is therefore a close relationship between these inorganic compounds and bone salts, such that theoretically both should be biocompatible. The vascularity and ingrowth of new bone is determined by the pore size of the ceramic (de Groot 1980) and

ceramic particles are phagocytosed by host cells. Bhaskar et al (1971) implanted tricalcium phosphate ceramic into the rat tibia and showed profuse ingrowth of connective tissue into the periphery of the ceramic. These results were verified in dogs (Levin et al 1974) by filling periodontal pockets with the ceramic and observing the material being removed by multinucleate giant cells and later replaced by bone. Similar findings were reported by Mors & Kaminski (1975) when clefts were treated in the dog palate. Radiologically the graft resorbed and later histology showed implant replacement by bone.

Hydroxyapatite synthetic implants have been shown to be biocompatible and if the pore size of the ceramic is suitable to allow the implanted area to be oxygenated, then new bone has been seen to grow up to and just into the periphery of the material (Nery et al 1975). However when implanted into the rat tibia, new bone is seen adjacent to the implant and there is no implant degradation at six months post-implantation (Denissen et al 1980). Unlike tricalcium phosphate which is a brittle ceramic, apatite ceramics are able to withstand high compressive forces.

Improvement in the properties of the ceramic materials is claimed with resorbable polyphasic calcium aluminate ceramics. When implanted in rhesus monkeys the material was impregnated and replaced with mineralised tissue (Graves 1972). By altering the manufacturing process of the ceramic, a wide range for times of degradation can be achieved without loss of the strength of the material. The performance of the ceramics has been compared by Uchida et al (1984) and Nade et al (1983). In the former paper,

implants were placed in skull defects in rats and rabbits. All the materials used were biocompatible and tissue ingrowth was found in pores of all implants but the depth of penetration was less for the aluminate ceramics than the apatite or tricalcium phosphate. No bone growth was found inside any of the ceramics. In the latter paper, marrow was placed with the ceramics in an inter-muscular site in the rabbit and newly formed bone was found to be strongly adherent to all the ceramics. Although all these materials are biocompatible, they do not appear to be biodegradable, at least in the short term.

Metallic implants for reconstruction of segments of the mandible have also met with varying degrees of success. The metals most commonly used are titanium, stainless steel and cobalt chrome alloys. Replacement of lost mandibular segments, including the condyle, with preformed titanium bars including a replacement for the condyle has been advocated by Bowerman & Conroy (1969). In what was entitled a preliminary report, they advocate the immediate replacement of any resected segment with the prosthesis. Their report is enthusiastic but only quotes two clinical cases with a follow-up time of only a few months.

It is considered that titanium and titanium covered implants are biocompatible with bone and Albrektson et al (1981) suggests the possibility of a direct bone to implant interphase contact. At electron microscopy, it was shown that titanium was bordered by a 20 nm thick layer of proteoglycans characteristic of ground substance. This separated bone from the implant surface; cells at the interphase were observed to be likewise separated while hydroxyapatite crystals were observed to be within this ground

substance layer (Linder et al 1983). This close apposition results in a sound mechanical lock when perforated titanium screws are used to hold a metallic reconstruction plate or prosthesis to bridge defects in the mandible (Raveh et al 1984). There is such good apposition in the cases quoted, that to remove the metallic support when the gap had been bridged, the heads of the screws required to be fractured off. Although the perforated hollow screws were left, the authors suggested that all plates and screws should be removed after bony union.

Stainless steel mesh trays have been used to support cancellous bone grafts for mandibular reconstruction (Salzer et al 1977). Three failures only were quoted in the 52 cases reported. Apparently none of the successful tray implants required to be removed. Terz et al (1978), using a stainless mesh prosthesis in a series of 102 patients, suggests the failure rate may be as high as 50% especially in areas previously treated with radiotherapy. Most prostheses seem to be lost due to subsequent exposure of the plates. There are long term follow-up reports of patients with metallic prostheses and Markowitz et al (1979) quotes a very successful 10 year result of a hemimandibulectomy patient with a prosthesis which included a temporomandibular joint section. The tissues around the metal implant in this case were not subjected previously to radiotherapy and the mandible was excised because of fibrous dysplasia. This result is probably not directly comparable to mandibulectomy for tumour, particularly if the area had previously been irradiated.

Metallic implants in the mandible therefore can provide stability and also continuity. If the prosthesis is in the form of

a perforated or mesh tray containing autologous cancellous bone or marrow grafts, it will also provide a support to increase graft persistence and allow new bone to be deposited in it.

The filling of bony cavities and improvement of bony contour has been treated over the last three decades or so by a variety of other foreign implant materials which it was hoped would be biocompatible and biodegradable.

Polymers have had a varied career. The early polyurethane polymers of the 1950s proved disastrous due to their cytotoxic effects (Redler 1962). Improved results were claimed for deeply buried polyethylene implants used as bone onlay grafts. It was claimed they were non-reactive and elicited no foreign body reaction (Rubin et al 1971) and finally that a fibrous capsule was formed around the material. The author's own experience with Silastic, a silicone polymer, is that if buried deeply, a membranous fibrous isolation barrier is formed around the material. If placed more superficially when it is used, for example as a replacement for the lost orbital floor in a blow-out fracture, most eventually become extruded. Recently interest has been shown in polyglycolate and polylactate polymers. Both are biodegradable and work has been started implanting these materials in varying proportions into bone (Miller et al 1977). Although lacking strength, these materials can be reinforced with carbon fibre to increase their durability.

There are therefore a great many materials used as a scaffold for lost bone, from the minor losses seen with periodontal pocketing to gross loss after malignancy surgery. None, except the vascularised and free autogenous bone transplants have the ideal

properties of a bone replacement material; total biocompatibility with complete, controlled biodegradability resulting in total replacement by host bone. Therefore a study of biological materials such as collagen, which is the main protein constituent of bone, could offer another approach to finding suitable replacement material for lost bone.

4. THE STRUCTURE OF COLLAGEN AND ITS ROLE IN BONE MORPHOLOGY

Collagen is phylogenetically a young protein present only in multicellular animals. The number of acceptable mutations of its amino acid composition is restricted by the physical requirements of the secondary structure (Pikkarainen & Kolonen 1969).

Collagen is synthesised in the fibroblast in the form of a precursor molecule procollagen. This molecule carries carboxyl and amino terminal extensions which are later removed by separate procollagen endopeptidases (Blobel & Doberstein 1975) to produce the primary unit of collagen, the tropocollagen molecule. Tropocollagen in the form of a triple helix is transported to the cell membrane where the end chains are cleaved and the molecule expelled (Feschler & Feschler 1978).

Tropocollagen is composed of three coiled amino acid chains. Each individual chain has a left-handed helical conformation and then like a three-stranded rope, the three chains are twisted around a common axis with a right-handed coil (Ramachandran & Kartha 1955). The three polypeptide chains each contain about 1000 amino acid residues (Cowan & McCravin 1955) wound in the triple helix with short non-helical extensions of 5-25 amino acid residues at both the carboxyl and amino ends of the chain.

Collagen chains from all species examined have compositionally common features; at least one third of the amino acid residues are glycine and about one fifth are proline or hydroxyproline (Rich & Crick 1961).

Five genetically different collagen types are now recognised. Type I, the first discovered, is found in bone, tendon and skin, it is also the major component of rat and kangaroo tail (a major source of research material). It contains two similar polypeptide chains and one dissimilar and is given the formula $[\alpha 1(I)]_2 \alpha 2$.

Type II collagen is unique to hyaline cartilage and vitreous humour and has a chain composition $[\alpha 1(II)]_3$.

Type III is found in skin, blood vessels and reticulin fibres, the chain composition being $[\alpha 1(II)]_3$.

Collagen in basement membrane is termed type IV and the collagen found in chorionic and embryonic membranes initially and later also found in skin, synovial membranes, gingiva and tendons is termed type V. The availability of types I and III has meant they have been studied most.

Epstein et al (1971) have given the amino acid residue composition of type I collagen (Table 1.1). These values for type I collagen compare favourably with that given by Eastoe (1955) for human bone collagen. Therefore all type I collagens seem very similar and the quantities of proline and hydroxyproline are essential for stabilising the helical structure. The non-helical terminal ends of the chains, by virtue of their lack of hydroxyproline and low glycine content, are unable to assume the helical configuration (Kang et al 1967). The function of the carboxy terminal extension may be to facilitate the folding of the

collagen helix during biosynthesis (Bornstein 1974) and the primary function of the amino terminal extension is to confer a high stability on the triple helix (Horlein et al 1979). This area is very resistant to heat and chemical denaturation. It is only after the removal of 30% of the amino and carboxyl terminal sequences by enzymatic digestion, that the tertiary structure is lost. A further function for these extensions has been shown by Wiester. et al (1979) who suggest that they are specifically inhibitive to type I collagen synthesis.

Types I and III collagens contain small amounts of carbohydrates, glucose and galactose in the helical portions of the molecule and they are attached to hydroxylysine residues. It has been suggested that the carbohydrate residues may regulate the formation of cross links or lateral aggregation of the collagen molecules into the quarter stagger arrangements (Light & Bailey 1981). There are two physiologically important areas within the triple helix that are concerned with inter molecular cross linking and they are the only examples of a sequence repeat within the α chain (Fietzek et al 1977). These regions can react with residues on the non-triple helical extensions to form cross links (Light & Bailey 1981). The possible locations for cross linking are numerous but highly specific and the cross linking enzyme lysyl oxidase only acts at specific lysine residues (Light & Bailey 1981).

There is much discussion about how the tropocollagen molecules are organised into the long striated fibrils seen on electron microscopy (E.M.) By X-ray diffraction, a periodicity for dry fibres of 640\AA is demonstrated which corresponds with the periodicity reported by E.M. using solubilised collagen. This

TABLE 1.1

Amino acid composition per 1000 residues in
 type I collagen (Epstein et al 1971) and
 human bone collagen (Eastoe 1955)

Amino Acid	Type I Collagen		Human Bone Collagen
	α 1 chain	α 2 chain	
Glycine	352	350	319
Proline	139	123	123
Hydroxyproline	102	87	100
Alanine	121	113	113
Glutamic Acid	74	69	73
Arginine	51	53	47
Aspartic Acid	43	46	47
Serine	39	36	36
Lysine	31	23	28
Valine	21	34	24
Leucine	21	33	25
Threonine	17	19	18
Phenylalanine	13	12	14
Methionine	7	5	5
Isoleucine	7	15	13
Hydroxylysine	5	9	3
Tyrosine	2	4	4
Histidine	2	11	6

banding pattern is characteristic even though the macromolecular structure of the tissue may vary, i.e. the collagen lattice becomes calcified in bone, which differs from type I collagen in skin.

Bone is a complex biological tissue composed of cells and intercellular substance. The intercellular matrix is composed of collagenous, elastic and reticulin fibres and a ground substance whose main components are glycosaminoglycans, protein polysaccharides, non-collagenous proteins and lipid either existing alone or in juxtaposition to each other. Ground substance is difficult to preserve during storage and sample preparation procedures (Frasca et al 1981). Chondroitin sulphate and sulphated protein polysaccharide are among the biochemical compounds that comprise it and it appears as a smooth amorphous material on E.M. scanning.

Collagen fibrils in bone are highly elongated and approximately circular in cross section, their diameter ranging from 80\AA to 4000\AA (Parry & Craig 1978). The fibres are composed of fibrils and bundles of fibres are visible by light microscopy. The fibre bundles are seen to branch out and interconnect with other bundles (Boyde 1972) and the smaller connecting fibre bundles may branch either perpendicularly or obliquely from the main fibres (Frasca et al 1981).

As collagen matures, the proportion of cross links decreases in most connective tissues to less than 10% of that found in the young animal. As they age, the fibres become increasingly less soluble and less susceptible to proteolytic enzyme action, thus becoming stiff with a decreased tensile strength (Vogel 1976). This is also seen when prepared collagen is artificially cross-

linked with more than an optimum number of linkages, when it also becomes very brittle.

In adult bone 70-80% of the mineral is located within the intermolecular spaces between fibrils and it is postulated that bone collagen acts as an in vivo catalyst for mineral deposition (Katz & Li 1973) during growth, repair and remodelling. Many of the properties attributed to collagen could therefore support its uses as a scaffold to aid bony repair.

5. COLLAGEN AS A BIOMATERIAL FOR CLINICAL USE

Collagen, as a major constituent of structures rich in dense connective tissue, such as dura mater, deep fascia or intestine (catgut), has been used as a natural product in various applications in surgery for almost a century (Chvapil 1977). For clinical use, collagen as a biomaterial is dependent upon its capacity for modification. To increase its tensile strength it must be made less soluble and this can be achieved by increasing the number of cross linkages in the molecules. When dispersed in acid solution, the original cross linkages in natural collagen are broken and when used in a reconstituted form, replacing and increasing the cross links restores the unique properties of the original fibres. This is the art of "tanning" (used in leather manufacture for more than a century) and cross linking is achieved with chromium sulphate or aldehydes such as glutaraldehyde, used under conditions of controlled temperature and pH. Thus the extent of tanning will control the rate of collagen resorption.

The material must be sterilised in such a way that its properties are not altered. Moist heat (autoclaving) will denature

collagen but gas sterilisation with ethylene oxide and γ irradiation from a cobalt 60 source will sterilise collagen for medical use with only slight denaturation (Chvapil et al 1973).

The rate of resorption of collagen will also depend upon the site of implantation and the local blood supply. There is a difference of opinion concerning the ultimate fate of implanted collagen or catgut sutures. Salthouse et al (1969) showed that there was a macrophage infiltration around catgut sutures. When used as a vascular prosthesis (Krajicek et al 1967) or as a collagen sponge (Chvapil 1977) it was suggested originally that the entire graft was gradually replaced by host tissue. Other workers including Potenza (1962 & 1964) have found that some implanted collagen is preserved and recellularised.

Current theory would suggest that collagenase is the agent responsible for the degradation of collagen and in recent years a number of mammalian collagenases have been detected in a variety of tissues (Harris et al 1969; Harris & McCroskery 1974; McCroskery et al 1975; Weeks 1976; Erlich et al 1977). The enzyme appears to be produced by a number of cell types (Lazarus et al 1968; Pelletier et al 1977; Turto-Lindy et al 1977). Collagenases examined so far appear to cleave the triple helical structure at or near the same locus, some three quarters of the way from the amino terminus of the α chain (Davison & Brennan 1982).

Mouse and rabbit fibroblasts and macrophages from many sources have been cultured independently and together; the cultured macrophages varied greatly in their potential for collagen degeneration. The fibroblasts showed a far greater potential for collagen digestion than the macrophages alone. In combination the

macrophages secreted a factor, a monokine that activated collagen destruction by the fibroblasts. The latter are therefore thought to be the major effector cell active in connective tissue degradation occurring in chronic inflammation (Laub et al 1982). Collagenase is secreted from the fibroblast as a combination of two zymogen granules which combine to become the active enzyme (Welgus et al 1980). Mineralised collagen can only be fragmented by collagenase when the inorganic phase has been removed (Krane 1982).

Suppression of collagenase activity is considered to be effected by the production of inhibitors by the fibroblast (Krane 1982). Other factors which control collagenase activity are the primary structure of the collagen molecule, type II showing a slower rate of degradation than types I and III. Cross linking of the molecules also greatly influences the extent and the rate at which collagen is denatured (Chavpil 1977; Krane 1982).

Collagen has been produced in various physical forms for surgical application - a solution, gels, films, membranes, sponges and tubing. In an extremely pure form, collagen is used as a plasma expander or to replace vitreous humour. Collagen in the form of a powdered fleece is considered an efficient haemostatic agent (Avitene™). In the form of films, membranes and tapes it has been shown to have a supporting role and it is used as a temporary wound dressing (Zenoderm™). In the form of fibres, collagen is still widely used as a resorbable suture material. Until the 1960's there was little information on collagen applicable to bone surgery as osseous replacement was mainly with the inorganic materials previously described.

Colago et al (1965) implanted a collagen powder extracted from the deep flexor tendons of cattle into the rat tibia and dog rib. He reported complete absorption of the collagen implant within 14 days and showed no difference in the rate of healing of collagen treated compared to control sites. There was however a significantly higher incidence of complications seen in the treated groups. It was concluded that collagen exerted little or no beneficial effect on the healing of bony wounds but the implant was very soluble and probably easily removed. The method of preparation did not include reconstitution of cross links, thus the material would be expected to be resorbed rapidly.

A report by Shoshan & Finkelstein (1970) concerning the effect of collagen on wound healing was more encouraging. They implanted collagen into the guinea pig lumbar muscle and claimed that in the early phase of wound healing, swelling, haemorrhage and inflammation were reduced and that mature fibrocytes were present within the labelled collagen implant by the sixth day. At 21 days, both the implanted and control sites were completely healed. The material used was a poorly cross linked homograft and no indication was given about the numbers of animals used in the work.

However, this view of a positive effect on wound healing was supported by Cucin et al (1972). They used a poorly cross linked lyophilised collagen preparation and implanted the material into resected rib sites in the rabbit. The periosteum was carefully retained and replaced during the operation. Radiographically they showed a more dense abundant callus at the implantation sites compared with the controls. Histologically a decrease of 30%-40% of the implanted collagen mass was noted at seven days but the

implants became revascularised and were shown to be included in the granulation tissue and callus before being replaced. This study concluded that no adverse reactions to the implant were seen and the material positively aided bone reconstruction. Only 11 animals were used in this study.

A collagen arterial graft of bovine origin was available in the 1960's which was highly cross linked. Devore (1973) compared this material cut into small segments with aldehyde cross linked calf skin collagen and non-cross linked calf collagen. The implants were placed in a resected area of the lower border of the rabbit mandible. The highly cross linked graft was readily accepted with little adverse reaction seen clinically or histologically. It seemed to become united to bone then gradually resorbed with bony invasion subsequently. The non-cross linked calf collagen was completely degraded and replaced with fibrous tissue by four weeks and although degradation of the aldehyde cross linked collagen was progressing it was at a much slower rate. New bone formation was noted throughout the collagen grafts.

This work was later extended (Devore 1977) to investigate the rates of degradation, and subsequent replacement with host bone, by bovine collagen cross linked to varying degrees by aldehydes including formaldehyde and glutaraldehyde. The rabbit mandible was again used as the site for implantation. At one and two weeks, the implanted sites remained unchanged and no fibrous tissue was found in the control areas. At two months, bone was seen to be permeating the formaldehyde grafts whilst others became encapsulated. At three months in the formaldehyde treated group, the operated areas seemed to be replaced almost completely by bone

whereas the highly cross linked glutaraldehyde treated implants remained intact and some became extruded below the lower border of the mandible. At one year, the control group had filled in the surgical defects with notching of the lower border of the mandible, whereas normal contour was regained in the formaldehyde group. The glutaraldehyde treated sites still showed evidence of the graft remaining at one year but with complete graft to bone adaptation and no loss of graft contour. In isolated areas, new bone was formed within this graft away from any areas of host bone. It was assumed therefore that the collagen materials placed into the defects initiated or aided the total replacement of lost bone and the greater the degree of cross linking, the longer the time for degradation.

Cochrane & Hiatt (1975) created defects in the iliac crest and femurs of dogs and filled test sites with microcrystalline collagen (AviteneTM). When compared with untreated sites the implanted areas showed variations of normal healing. Some sites filled with fibrous tissue alone, whilst others filled with bone. There was, however, no detectable acute foreign body reaction to the implant and no residual material could be identified at any site after three weeks. Whilst it was concluded that the implant had no detrimental effects on the cellular repair of bone, there is no indication from this work that any positive effect was seen.

Hunt & Benoit (1976) evaluated the same material (AviteneTM) in the post-extraction tooth socket in the cat. They found that it greatly improved wound haemostasis but the acute inflammatory reaction around the sockets was more severe and extensive when compared with the control sites. Although not commonly found,

foreign body giant cells were seen resorbing the collagen fragments. Healing was delayed in the treated sockets at two weeks but by four weeks all the implant had been removed. Healing in both treated and untreated sockets showed new bone formation by six weeks but in the central region of the treated sockets, the bone was sparse. This work concluded that the effect on haemostasis was beneficial but a denser, longer lasting reaction with a delayed healing time was evident. A retardation of healing was also observed by Debalso & Adrian (1976) implanting collagen in tibial defects in guinea pigs and Moskow et al (1976) implantating sites in the rabbit femur.

An opposite view is held by Joos & Ochs (1980) who imply that implanted collagen may calcify directly and quickly lead to new bone formation. They claim that bone formation is induced by collagen, which is seen initially as mineralisation of acellular collagen later followed by cellular migration of osteoblasts and fibroblasts to form bone spicules which coalesce within the implanted area.

Krekler et al (1980) support such a view with their work on bony pockets in beagle dogs. They further claim that marginal bone grows into the collagen fibres and thus bony regeneration is accelerated. A human study by the same authors (Fabinger et al 1980) placed the material into periodontal pockets. In these sites it proved difficult to keep in place and close apposition between bone and implant was lacking but they still claimed an increased bone growth of 32%-71% in selected bony pockets. No time scale is given in this report and long term follow-up of patients is not commented upon.

Further human studies implanting collagen into cyst cavities and bony defects in the mandible and maxilla have been performed (Fabinger et al 1983; Zetzmann et al 1982). Both series report highly favourable results with implanted collagen for bone regeneration compared with control sites, results were monitored radiographically. A late reaction, possibly to the implanted material, of a lymphonodular foreign body reaction two years after implantation is reported by Zetzmann et al (1982) as the only complication found in the series.

Further animal work using glutaraldehyde cross linked collagen and collagen mixed with apatite particles was reported by Hayashi et al (1982). The resected rabbit fibula was used as the implantation site. They showed that in the cross linked collagen group, inactive osteogenesis was seen around the implant with no ossification activity invading the collagen and a new bone only being formed around the implanted material. More vigorous osteogenesis was seen in both the control group and the collagen with apatite implant group; repair in the latter group was reported as being more advanced than the control and no loss of bony contour was eventually found.

The authors concluded that ossification was inhibited in the cross linked collagen implanted defects and did not support the earlier findings of Cucin et al (1972) and Devore (1977) that foreign body reactions were rarely seen and that ossification was enhanced by the collagen implants.

It would seem that opinion is divided on the merits or otherwise of collagen as a biomaterial to assist in the healing of bony wounds. Most authors agree that the material is biocompatible

but there are obvious differences in the degree of cross linking of the collagens used and therefore in the behaviour of the implant. Several species of experimental animal have also been used, there are variations in the sites chosen for implantation and different sources have been used to obtain the collagen implant materials. These differences may in part account for the variance of the results obtained. A suitable material would therefore be one which was cross linked to allow for a prolonged resorption but not so highly cross linked that it was physically brittle and could not be resorbed.

6. IMMUNOLOGY OF COLLAGEN

Knowledge concerning any implanted protein is incomplete without an account of its immunological reactivity. Much controversy has arisen on this subject concerning collagen. Initially the problems were thought to be due to the lack of "purity" of the substrates used since the presence of even small amounts of contaminants such as serum proteins have been shown to produce antibody formation (Engel & Catchpole 1972).

In 1930 Loiseleur & Urbain first tested the ability of an acid soluble collagen from rat tail tendon to induce antibody formation in rabbits. Antibodies were produced and demonstrated using complement fixation. The collagen preparation seemed poorly purified and it was difficult to ascertain which antigen was being demonstrated.

In 1949 Waksman & Mason, using a chemically "purified" human and rabbit collagen free from extraneous protein and lipid, injected it into rabbits. No complement fixing antibodies nor

precipitating antibodies could be demonstrated and it is arguable that the crude method of purification may have affected the antigenicity of the injected material.

Repeating the earlier work, Watson et al (1954) using acid soluble rat tail tendon collagen and Schmitt et al (1964) using calf collagen, showed a weak antigenic response in the rabbit by complement fixation. The early work was thus conflicting but now the picture is becoming a little clearer.

The primary structure of collagen and chains has been elucidated (Mark 1981) and it is seen that the species variation in the amino acid sequence of the helical portion is minimal. This area shows a high degree of evolutionary stability and more variability is seen in the terminal non-helical portions.

Anti-collagen antibodies have been raised in rabbits, rats, mice, guinea pigs and chickens and a variety of routes of immunisation used. The methods of detection used, include complement fixation, immunofluorescence, radioimmunoassay and immuno-precipitation. It has also been shown that the nature of the immune response does vary between species and the immunogenicity of a whole and a denatured collagen preparation varies considerably (Michaeli et al 1971). Denatured bovine type I collagen was found to be non-immunogenic in mice but a good response was found with the natural form (Nowack et al 1975). A similar bovine collagen injected into the rabbit shows no difference in immunogenicity between the native and denatured state (Steffan et al 1968).

Protein molecules may be separated into antigenic determinant areas and immunosilent regions (Sela 1969). In the collagen

molecule there seems to be two categories of antigenic determinant. Firstly, a linear amino acid arrangement, i.e. a non-conformational determinant and secondly, a conformational dependent determinant which reacts only in the native molecule and not the denatured collagen (Sela et al 1967). Rabbits produce conformational dependent complement fixing antibodies to calf collagen (Schmitt et al 1964) but conformational independent antibodies to rat collagen (Timpl et al 1972).

In 1964 Schmitt et al postulated that for collagen the major antigenic sites were located at the terminal non-helical regions. Steffan et al 1968 demonstrated three different sites for rabbit antisera to bind with bovine collagen, two were pepsin stable and one pepsin labile, thus concluding that part of the immunological response was in the helical and part in the non-helical region of collagen molecule. However, pepsin cleaves only the C terminal end of the protein (Beil et al 1973) and protease did not affect the antigenic activity of collagen in mice (Nowack et al 1975). Further evidence was needed to determine the specific antigenic sites as it was also known that collagen degradation by collagenase, trypsin or other proteases produced peptides that still retained or had a greater antigenic potency (Kirrane & Robertson 1968).

A more successful approach towards the localisation of the antigenic sites in collagen was shown by Michaeli et al (1969) using cyanogen bromide peptides (CNBr) derived from individual α chains to show antigenic determinants in non-helical segments and non-conformational sites.

Radioimmunoassay studies on CNBr peptides (Lindsley et al 1971) showed that the major antigenic determinant of the α chain of rat skin collagen was at the C- terminal region but there was a late appearance of antibodies directed also to the N terminal site of the α chain (Timpl et al 1972). It would also appear that each antigenic site in the non-helical region is unique for collagen of a given species.

Antibodies to central helical sites show a high degree of cross species reaction (Timpl et al 1971). Calf collagen antigen injected into the rabbit shows the major antigenic site is in the terminal non-helical peptide chains but the helical portion does act as a minor antigenic site (Michaeli et al 1969). If the same collagen is injected into the rat, the major site of antibody production was against the helical portion with the terminal sites not being involved. These antibodies did not react with the denatured molecule but when returned to the triple helical structure, there was recovery of antigenic activity (Beil et al 1973).

The conclusion from the available literature is that antigenic sites on the collagen molecule depend on the origin of the collagen injected and the species of animal used. They may be predominantly terminal or helical in origin. However, according to Timpl (1976), insoluble cross linked collagen is substantially lacking in immunogenicity. Other biological parameters may also contribute to the successful production of antibodies. Apparently different immune response genes govern the optimal production of antibodies against type I collagen, type III collagen and procollagen. The control of the immune response to collagen requires co-operation between thymus dependent T cells and bone marrow derived B

lymphocytes. Nowack et al (1976) showed that response to native calf collagen was T cell dependent in mice but Fuchs et al (1974) demonstrated thymus independence of a collagen like synthetic polypeptide and rat collagen in mice, but the need for both T and B lymphocyte co-operation in the immune response to gelatin. Several different genes may therefore be involved in the control of the antibody response to collagen.

In vivo the cell mediated response to collagen was studied by Grillo & Gross (1962). Implants of calf skin collagen were injected into rabbit toepads in Freund's complete adjuvant. The 24 hour response to injected and control animals was similar. Implanted animals showed a slight rise in reactivity at one week which fell to its initial level at six weeks. It was felt, however, that the collagen was impure and if prepared pure, would not excite such a reaction.

The experiment was repeated by Adelman et al (1972) who showed that a delayed type hypersensitivity reaction was maximal at 20 days after immunisation and persisted for a further three weeks. The skin reactions were species specific and always greatest against the initial immunizing agent, but cross reactions were readily obtained with other mammalian collagens except in the guinea pig experiments. Guinea pigs given homologous or heterologous collagen showed no cell mediated reaction at all. The ability of the individual chains to induce cell mediated reactions had also been shown. All individual chains were capable of inducing cell mediated immune reactions with considerable cross reactivity but the strongest reactions were against the initial immunising agent indicating some degree of species and chain

specificity.

Adelmann & Kirrane (1973) using pepsin digested collagen showed no change in strength of species specificity of the reactions and indicated for cell mediated immunity the determinants probably lay in the helical region of the molecule. After heat denaturation of the helical structure, cell mediated immunity could still be induced but there was no subsequent reaction when the native material was used as the challenge agent Adelmann (1972). Thus it would appear from the evidence that conformation may be of significance in the induction of a humoral and a cell mediated response.

Presently the consensus of opinion is that the intact collagen molecule is considered as a "weak" antigen but a single exposure can be enough to obtain a measurable antibody response. This level of response is said to remain essentially unchanged after subsequent injections (Kirrane & Robertson 1968). The original hypothesis for "weak" immunogenicity thought to be the homology between collagen molecules of different species (Kirrane & Glynn 1968) has not really been substantiated (Pawlowski et al 1975).

There are therefore many unanswered questions concerning collagen immunology. In particular, the genetic factors that govern "major" and "minor" antigenic sites which seem to depend on the species of collagen substrate and the recipient animal species; also to what extent the primary, secondary, tertiary or quaternary structure of collagen is involved in its immune response.

The immunology of collagen is therefore a debated subject. The material used in this thesis is highly purified and any immune response elicited by its use is assumed to be directly related to it.

7. ADVERSE EFFECTS OF COLLAGEN IMPLANTS

Several collagen preparations are now available for commercial use. Two such preparations, Avitene™ and Zyderm™ have been used clinically. Avitene™ is produced as a sterile water insoluble fleece and has been shown to be effective when used as a topical haemostatic agent in man and experimental animals (Hiat et al 1973; Alexander & Rabinowitz 1978; Evans et al 1979; Vistnes et al 1974 and Abbott & Austen 1974). Zyderm™ is a purified collagen suspension kept in a solution at 0-5°C of buffered physiological saline and a local anaesthetic. Its molecules thus remain suspended and separated. When the temperature of the material is brought to 37°C during its implantation in the dermis, the physical properties change. It undergoes fibrous transformation into an opalescent gel and then to an opaque semi-solid mass of orderly condensed fibrous collagen with the loss of water of hydration. This framework serves as a support for ingrowing vessels and cells to restore dermal tissue lost in acne scarring and by age in the majority of cases so far treated.

Bovine dermis is the source of collagen used in both preparations which has been partially digested to cleave the main antigenic determinants from the triple helix and produce a product of supposed low immunogenicity.

Little has been written concerning adverse effects produced by Avitene™ used as a topical haemostatic agent. When it was buried in the peritoneal cavity in a report by Park et al (1981), some doubt was caused about its totally benign nature. The material was used in conservative ovarian surgery as a haemostatic agent. Avitene™ was seen to have reacted as a foreign body when the

abdomen was reopened, an extensive granulomatous mass being found in the implanted area. The tissues may react differently when the material is buried in the abdomen.

There are conflicting reports concerning the disposal of implanted collagen. Oliver et al (1982) have demonstrated that transplanted dermal collagen is resorbed by a non-specific digestion with tissue collagenases rather than by cellular or humoral mediated immune responses. Using a range of homogenic, heterogenic and xenogenic collagen implants, they have shown that the collagen did not provoke an immune response but that unless the material was fixed with substances such as glutaraldehyde to protect it from collagenase digestion, then the implant would be resorbed. This view did not support the work by Trentham et al (1978) who suggested that in the rat model, homologous, heterologous or denatured collagens were capable of inducing both cellular and humoral responses.

In the general population it would perhaps be expected that some patients would be innately sensitive to injected foreign proteins and in others repeated injections of an allergen may produce sensitisation.

Over the first five years there have been a small number of reports concerning reactions to injected collagen either at a test site or an implantation area (Barr et al 1982; Brooks 1982; Swanson et al 1983; Cucin & Barek 1983; Barr & Stegman 1984). Such reactions were known and considered during the initial trials with Zyderm™ from 1979 to 1981. The information from the Collagen Corporation concerning these trials shows that over the two year period, 9427 patients received collagen implants and 5109 were



followed up. The majority were treated for age related dermal loss and acne scarring. Many had repeated implants to maintain the maximum correction achieved. All 9427 patients were given a test intradermal dose. Two hundred and eighty four patients (3%) developed a reaction at this site and 54 of them also complained of more generalised symptoms such as arthralgia, myalgia, pruritus or rash. Of the patients followed up, 1.3% (5109), developed implant site reactions after a negative test site reaction. A localised area of swelling, induration, erythema, urticaria or pruritus was seen. All such reactions were seen within the first four injections and it would appear they had become sensitised during their treatment. The incidence of adverse reactions is thus low, but significant.

Several further studies have served to clarify the situation with regard to the immunogenicity of bovine collagen implants.

Kramer & Churukian (1984) gave their results of a retrospective study from 1979 to 1982 of 300 patients. They found a positive skin test in 3.5% and local and systemic reactions to implanted collagen in 1.3%.

Cooperman & Michaeli (1984¹) selected patients who had reacted to the implants in a retrospective study. They found in this series that 31 patients had developed a test site reaction alone, 35 experienced generalised symptoms with no test site reaction and six reported both a local and a generalised reaction. The sera of all these patients was examined for anti-implant antibodies by a radioimmunoassay technique.

Significant correlation was found with patients who had developed local reactions showing elevated levels of antibody to

Zyderm[™] collagen. Systemic complaints could not be correlated with antibody titres. As well as this retrospective study the authors had undertaken a prospective study (Cooperman & Michaeli 1984²). Two of 61 subjects (3%) experienced a self limiting inflammatory response to implanted material and in only these subjects could elevated levels of anti-implant collagen antibodies be demonstrated. It was also shown that these antibodies did not cross-react with human dermal collagen nor did they result in elevated levels of circulating immune complexes. The reacting areas were biopsied and showed a mixed inflammatory infiltrate (neutrophils, eosinophils, lymphocytes and histiocytes) found predominantly around vessels and foci of the implant. Fibroblasts were found within the implant material. The clinical response reported by the two subjects was prior to elevated antibody levels. Elevated levels were first measured at the six month follow-up period.

If collagen is a T cell dependent antigen and the response depends on appropriate presentation of antigen to T cells, then it may be that localised cell mediated reactions could precede the presence of detectable anti-implant antibodies.

From the information available so far it could be concluded that the anti-collagen antibodies are specific for the type of collagen implanted and probably should not cross react with human collagen. It is still wise, in the present state of knowledge, to exclude patients with autoimmune diseases, those with a history of anaphylactoid reactions or pregnancy from any studies with collagen implants. It has also been shown that, in the experimental animal, when autoimmune disease has been attributable to collagen antibodies, it is type II collagen that has been implicated; no

reference has been made to an adverse immunological response to type I (Trentham et al 1978; Cremer et al 1983).

CHAPTER 2

THE EFFECT OF A HETEROLOGOUS COLLAGEN MATERIAL
ON BONE HEALING - THE AIMS OF THIS STUDY

The aim of the study was to investigate the effects and subsequent replacement by host tissue of the reconstituted collagen paste of bovine origin described in the introduction. The study used an artificial bony defect created in the rabbit mandible to demonstrate the effects on healing and then further considered the effect the material had when placed in bony cavities caused by disease in the jaws of man.

a) Wound healing and the inflammatory response

The effect the material had at a cellular level was demonstrated in vitro by its effects on human fibroblasts grown in tissue culture. The effects in vivo were seen by a longitudinal histological examination of healing bony wounds created in the rabbit mandible. Two comparable bony lesions were created, one used as a control area, the other implanted with the collagen paste. The early effects on bone healing as the osteogenic granulation tissue advanced were attempted to be shown by a study following the revascularisation of the surgical defect with a radiopaque dye injection technique. The effect the material had on calcification of the surgical defects was to be shown directly using an in vivo bone staining method, undecalcified sections and ultraviolet microscopy.

b) The immunological response

The immune response shown by the rabbit was viewed in the longitudinal histological investigation by examination of the cell types present during healing of the operated areas and the degradation of the bovine collagen matrix. Co-operation between T and B lymphocytes appears to be responsible for the humoral response shown to implanted collagen. Antibody directed against bovine collagen was demonstrated by an enzyme linked immunoassay (ELISA). Changing levels of antibody were monitored by weekly serum sampling from implanted animals.

c) Clinical applications of heterologous bovine collagen
in bony defects in man

To test the clinical safety and efficacy of heterologous bovine collagen, a human implantation study was used. Bony cavities caused by local disease in the maxilla and mandible were used as the sites for investigation. The jaws are useful as lesions of localised bone destruction up to 5 cm in diameter are common and easily accessible. A comparison of control areas and implanted sites was compared radiographically over a one year period.

CHAPTER 3

CLINICAL METHODS I. ANIMAL INVESTIGATION

Introduction

Draenert & Draenert (1980) have shown that in the rat tibia bony defects up to 800 μ in diameter will heal by primary intention, therefore by inference a larger area will heal only by secondary intention. The early work for this thesis had shown how difficult it was to follow healing by secondary intention in a defect in the rat mandible 1 mm in diameter and 3 mm in depth. To demonstrate healing potential, a bony lesion should therefore be of an effective size, be easy to duplicate in each animal and able to be identified later.

Several authors (Nathanson 1977 and Devore 1977) have used the lower border of the rabbit mandible as an implantation site. The lower border is of the order of 5 mm in width below the molar teeth and at this site an area of 10 mm x 5 mm can be removed with ease via a submandibular approach. This area is therefore constant and comparable defects can be made in each animal. Unfortunately the roots of the molar teeth and the inferior dental canal will be severed when this area is removed. The available data from the previous studies indicates that no untoward effect is seen to the molar teeth in the mandible itself. This site was thought to be suitable, because of easy access, to cut a bony cavity so that a substantial quantity of implant material could be buried.

a) **Operative Surgery**

i) **Animal allocation**

Thirty one young adult female New Zealand white rabbits approximately six months old and weighing 2.46 Kg to 3.62 Kg (average 3.08 Kg) were operated on. Originally 30 animals were to be used, being divided into 10 groups of three, but one animal died under anaesthesia and was therefore substituted to make the total of 31.

Each animal was allocated a unique number and each group of three animals allocated a group number. The group number signified the survival time. Groups 1, 2, 3 and 4 were sacrificed at three, five, seven and 10 days respectively. Groups 5, 6, 7, 8 and 9 were sacrificed at weekly intervals from two to six weeks and animals in Group 10 survived for 12 weeks.

One animal from each of Groups 3 to 10 inclusive was given tetracycline and haematoporphyrin for bone labelling between operation and sacrifice. The remaining two animals in each of these groups were used for routine histological examination and mandibular radiology. One animal designated A2 from Group 5 (14 day survival) was used to demonstrate the microcirculation by the radiopaque dye injection technique. Two animals, A27 and A32, from Groups 9 and 10 (42 and 84 day survival) were used in the immunological study. Serum samples were taken from A32 from weeks 0 to week 6 and from A27 from week 7 to week 12 inclusive. Two animals were used for the 12 week period, as repeated venepuncture at weekly intervals for six weeks caused considerable damage to the ear veins of the first animal. The use of both animals resulted in continued successful venepuncture (Table 3.1).

TABLE 3.1

ANIMAL ALLOCATION

Group/ Survival	Animal No.	Light Microscopy	Fluorescent Microscopy	Radiology	Immunology	Microcirculation
(5) 14 days	A1 A2 A3	 + +	 + 	 +		 +
(6) 21 days	A4 A5 A6	 + +	 + 	 +		
(3) 7 days	A7 A8 A9	 + +	 + 	 +		
(4) 10 days	A10 A11 A12 A13	 + +	 +	 +		
(8) 35 days	A14 A15 A16	 + 	 + +	 +		
(7) 28 days	A17 A18 A19	 + +	 +	 +		
(1) 3 days	A20 A21	 +		 +		
(2) 5 days	A22 A23 A24	 + +		 +		
(10) 84 days	A25 A26 A27 A28	 + +	 + 	 +	 +	
	A29	Unsuitable for use on delivery.				
(9) 42 days	A30 A31 A32	 + +	 + 	 +	 +	

ii) Procedure

The anaesthetic agents used in the experiment were:-

Hypnorm™ (Fentanyl citrate 0.315 mg/ml with Fluanisone 10 mg/ml) and Diazepam™ injection B.P. containing 5 mg Diazepam™ per ml).

The animals were firstly anaesthetised with Hypnorm™ being given intramuscularly in a dose of 0.2 ml/Kg body weight then when drowsy they were given Diazepam™ intravenously into a vein in the ear in a dose of 1 mg/Kg body weight. If the animal was not fully anaesthetised and reacted during hair clipping, a further dose of Diazepam™ of 0.5 mg/Kg was given intravenously before the operative procedure began.

The fur was shaved under both sides of the mandible from the trachea to the symphysis menti with electric clippers.¹ The animal was turned firstly into the left lateral then right lateral positions for operating on the respective sides of the mandible. The freshly prepared skin was cleaned with 70% isopropyl alcohol. An incision was made through the cleaned skin using a No. 15 Swann-Morton blade between the two halves of the mandible from the symphysis menti distally to the trachea. As the tissues are lax, the skin wound was advanced over the lower border of the right mandible and the incision deepened under the body of the mandible separating the digastric and zygomatico-auricularis muscles. The periosteum was therefore exposed over its lower border, from anterior to the masseter muscle and facial vessels, to below the diastema region posterior to the incisor teeth. The periosteum was incised

¹ Harvard Bio-Science, Fircroft Way, Edenbridge, Kent.

and elevated from the underlying bone with a Howarth's periosteal elevator to expose an area of the lower border of the mandible of about 2 cm.

An electrically operated micromotor handpiece¹ with isotonic saline as a coolant and irrigation was used to drive a flat fissure burr to cut a rectangle of bone 10 mm x 5 mm from the lower border of the mandible across its entire width. All visible bone debris was removed by warmed saline irrigation and suction. The block of excised bone contained the contents of the inferior dental canal and the apices of the adjacent molar teeth and initially the area bled profusely. The rabbit mandible and a diagram to illustrate the segment removed are shown in Figures 2 and 3.

The collagen paste was expressed from the sterile syringe into the mandibular defect on the right side to completely fill the cavity and moulded into shape with the Howarth periosteal elevator. After haemostasis was achieved, the animal was turned and via the same skin incision, the left mandible was exposed by advancing the skin wound to the left side and a similar block of bone excised from the lower border. The resultant cavity on the left side was allowed to fill with blood clot.

The right mandible in all cases was implanted with collagen and the left side contained blood clot. When there was haemostasis in both operated areas, the periosteum and overlying muscles were closed with 4/0 polyglycolic acid (Dexon™) sutures and the single skin incision was approximated with a continuous 6/0 prolene suture.

¹ Quayle Dental Company, Dominion Way, Worthing, Sussex.



Fig. 2 Lateral view of normal adult rabbit mandible.

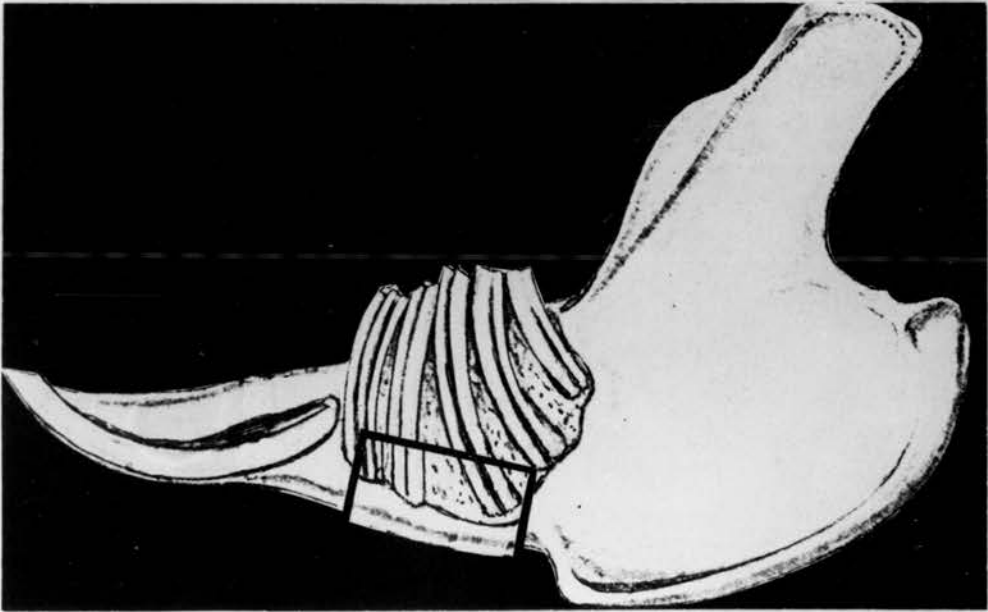


Fig. 3 Diagram to show site, size and shape of area of operation on lower border of mandible.

The animals were allowed to recover in the operating area then replaced in their cages and later fed on the standard pellet diet¹ and water, supplemented by cabbage leaves, until sacrifice.

**b) Microangiographic Study of the Microvascular System
in the Healing Bony Defect**

The report by Shoshan & Finkelstein (1970) suggested that collagen had a positive effect on wound healing reducing the tissue reaction in the early phase. Cucin et al (1972) also suggested a positive effect on wound healing by collagen. If the time taken for proliferation of osteogenic granulation tissue can be reduced, then a microvascular circulation should be seen earlier on the implanted side compared with the control area. It was therefore considered that one animal in each of Groups 1 to 6 inclusive (survival times 3 days to 3 weeks) should be infused with a radiopaque contrast medium to detect the time the microcirculation could be demonstrated in the implanted and control areas.

Microangiography, introduced by Barclay (1947) involves filling of a vascular system with a contrast medium. The medium should have a well defined particle size, small enough to fill the vessels to be observed, and have no tendency to aggregate. The reagent selected for this study was Hypaque™ (Hypaque sodium consists of 25% w/v sodium dinitrozoate B.P. The anhydrous material contains 59.87% iodine.) It was chosen as it was known to be safe for human use and had a suitable particle size.

¹ Special Diet Services Ltd., 1 Stepfield, Witham, Essex.

Method

The technique mainly followed that of Danckwardt-Lillieström (1969). The first animal to be used for this investigation was A2 (Group 5, 14 days survival time). At 14 days post-surgery the animal was anaesthetised again, as previously described, with Hypnorm™ 0.2 ml/Kg body weight intramuscularly then Diazepam™ 1 mg/Kg body weight intravenously and placed in a supine position. Both common carotid arteries were exposed in the neck and cannulated in the distal direction with gauge 32 intravenous cannulae. Under slow and gentle pressure, 10,000 U of heparin¹ (5,000 units of sodium heparin per ml in 8 ml N saline) was administered by way of a two-way extension set into both common carotid arteries over a five minute period.

A solution of Hypaque™ 25% (200 ml in 500 ml warm physiological saline) in a sterile container was suspended from a drip stand and then infused under a pressure of 200 cm of water over a 2 hour period via an intravenous infusion set to the extension set and into the cannulae in the common carotid arteries. Blood was withdrawn via the jugular veins to reduce the venous congestion. The animal survived for 10-15 minutes and the infusion was therefore mainly carried out after death. After infusion of the 700 ml of solution, the mandible and surrounding musculature were removed and the tissues frozen at -40°C.

After thawing, most of the soft tissue was excised carefully not disturbing the implanted and control operation sites. The denuded mandible was then bisected at the symphysis menti. Each

¹ Leo Laboratories Ltd., Aylesbury, Bucks.

half was placed on Ultraspeed D dental occlusal film. A Phillips Oralix¹ tube set at 50 kv was placed with a fixed focal distance of 18 inches from the film and the mandibles radiographed with an exposure time of 1.04 seconds. The focal distance was then reduced to 16 inches and subsequently by 2 inch intervals to 10 inches to try and find a suitable distance for optimal resolution in the operated area. The exposure time remained constant at 1.04 seconds.

c) Fluorescent Bone Labelling

Tetracycline labels are deposited as permanent markers within bone mineral at the calcification front in the presence of active mineralisation of new osteoid in the centres of new bone formation. If labelled bone is decalcified, the tetracycline is lost, so undecalcified sections must be used. This bone marker has been used for over 30 years and has been found to be safe and effective (Frost 1969). More recently, there has been a tendency to use more than one label to follow the continuous laying down of new mineral. Tetracycline produces a yellow band under ultra-violet light, haematoporphyrin² is probably fixed to new bone in the same way as tetracycline and shows red (Olerud & Lorenzi 1970).

One animal from each of Groups 3 to 10 inclusive was selected for this study, survival times were thus from one to 12 weeks. The animals used were A1, A4, A7, A10, A14, A18, A25 and A30. Animal A16 was substituted for A14 after the latter developed a wasting

1 Phillips Medical Systems, Earls Gate Lodge, St. Ninian's Road, Stirling.

2 Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset.

condition with ataxia and joint swelling after injection of haematoporphyrin and had therefore to be sacrificed at 3 weeks, not 5 weeks, as originally intended.

Table 3.2 confirms the animals used and their survival times.

The reagents used for in vivo bone labelling were Achromycin™ and haematoporphyrin.

Achryomycin™ consists of tetracycline hydrochloride 250 mg per vial buffered with 625 mg of ascorbic acid. The solution was reconstituted by the addition of 5 ml of water for injection immediately prior to use.

Haematoporphyrin as used as the hydrochloride; the solution was prepared as follows:- 3 g of haematoporphyrin H 1875 were dissolved in 100 ml of 2% sodium carbonate. Solubility was increased by adding alcohol in a concentration of 10% and warming the solution to 40°C. The solution was then filtered to remove any undissolved particles.

Immediately after operation, while the animals were still anaesthetised, 50 mg/Kg body weight of Achromycin™ was administered by intramuscular injection into the thigh. Seven days later, 10 ml (300 mg)/Kg body weight of haematoporphyrin was administered subcutaneously into the loose connective tissue of the neck posteriorly, over a period of 15 minutes. One week later, a further similar dose of Achromycin™ was given intramuscularly then haematoporphyrin one week later. This alternate weekly labelling was continued until seven days before sacrifice (Table 3.3).

For the long term surviving animals, A25 and A30, haematoporphyrin was not used because of the severe toxic effects found in animal A24, therefore only tetracycline (Achromycin™) given at two

weekly intervals on weeks 0, 2, 4, 6, 8 and 10 was used.

After sacrifice, all the mandibles were immediately harvested and deep frozen at -40°C . The animals used, their survival times and their times for bone labelling are shown in Tables 3.2 and 3.3.

d) Radiological Study of Healing Bony Cavities

New bone formation is said to be seen histologically as early as 7-10 days post injury when osteoid tissue is produced. Radiographically at this stage the quantity of calcified matrix is not sufficient to be detected by standard radiographic means. Much more calcium must be deposited before there is a change in radiodensity visible on radiograph. Earliest ossification will be seen peripherally as the osteogenic granulation tissue advances into the defect. Initially the sharply cut bone edge at the margin of the defect will become less distinct radiographically as the early multicentre ossification proceeds in an irregular fashion; then as the areas coalesce, a more distinct calcification front will be produced. Radiological assessment of the bony wounds is used to compare the degree of calcification in the implanted and control areas. This should be comparable with calcification seen in the fluorescent bone study. The information from this study will serve as a basis for the radiological assessment of the healing bony defects in the jaws in the human implantation study.

Method

After death, mandibles from animals in Groups 2-10 inclusive (Table 3.4) were removed as previously described, with some surrounding muscle, and bisected at the symphysis menti.

TABLE 3.2
Fluorescent Microscopy

Group	Survival Time	Animal No.
3	7 days	A7
4	10 days	A10
5	14 days	A1
6	21 days	A4, A14
7	28 days	A18
8	35 days	A16
9	42 days	A30
10	84 days	A25

TABLE 3.3

Administration of Fluorescent Bone Labels

Group	Animal No.	Weeks Post-operation								
		1	2	3	4	5	7	9	11	
3	A7	T								
4	A10	T								
5	A1	T	H							
6	A14, A4	T	H	T						
7	A18	T		T	T					
8	A16	T	H	T		T				
9	A30	T		T		T				
10	A25	T		T		T	T	T	T	

T = tetracycline

H = haematoporphyrin

TABLE 3.4

Radiology

Group	Survival Time	Animal No.
2	5 days	A22, A23, A24
3	7 days	A8, A9
4	10 days	A12, A13
5	14 days	A2, A3, A17
6	21 days	A5, A6
7	28 days	A19
8	35 days	A15
9	42 days	A32
10	84 days	A27

The bisected halves were each placed in turn on a Kodak Ultra-speed D dental occlusal film. A Phillips Oralix tube was used for the exposures which were taken with a fixed focal distance of 18" and an exposure time of 1.04 seconds at 50 KV.

The films were developed using M & B Matalex developer¹ and fixed with M & B fixer plus, by automatic processing in a Pantomat².

The intra-oral films were processed on a six minute cycle.

Radiological changes at the sites of operation were then compared.

1 May & Baker, Dagenham, Essex.

2 Siemens Ltd., Wheatfield House, 6 Wheatfield Street, Edinburgh.

CHAPTER 4

LABORATORY METHODS

a) A Longitudinal Histological Examination of the Inflammatory Response, the Immune Response and Wound Healing by Light Microscopy

All wounds show an acute inflammatory response followed by an immune response; examination of the changing cell population in the implanted area and control site will show the effect exerted by heterologous collagen. The duration of the acute inflammatory response is affected by the presence of foreign protein; as the acute inflammatory cell population diminishes it is replaced by mononuclear cells. In the immunologically mature animal contact with antigenic material not only leads to the production of antibodies but also to the development of a cell mediated response. This latter response characteristically follows the injection of an antigen into a previously sensitised individual and is seen to develop clinically over 48-72 hours.

Mononuclear cells, lymphocytes and macrophages make up the general cellular population in such a response and giant cells derived from the macrophages are seen to form around any large particles of foreign protein. When large particles are not phagocytosed by macrophages, the giant cells may form into a syncytial mass around the foreign body. Delayed hypersensitivity reactions have developed clinically after dermal injections of Zyderm™ soluble collagen in susceptible individuals.

This histological study will show the changing cell population and the time taken for incorporation or disposal of the collagen implant. The relative times taken for the appearance of osteogenic granulation tissue, formation of woven bone and the time taken for callus to fill the bony defect will be compared for the implanted and control areas to determine the total effect of the material throughout healing.

Method

The animals were sacrificed at the pre-determined times shown in Table 4.1 ranging from 3 days to 84 days. All animals were sacrificed by barbiturate overdose using Nembutal¹ given intravenously.

The mandibles and surrounding musculature were excised, bisected at the symphysis, clearly labelled and fixed in a 4% solution of phosphate buffered formaldehyde (10 ml of 4% formalin [37-40% formaldehyde solution] added to 90 ml of phosphate buffer) for not less than four days. The mandibles were then removed and rinsed for one hour in phosphate buffer (0.2 M solution of dibasic sodium phosphate $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ prepared with 71.64 g and distilled water to 1 litre. 0.2 M solution of monobasic sodium phosphate $\text{Na}_2\text{H}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ prepared with 31.21 g in 1 litre of distilled water. Phosphate buffer at pH 7.0 at 25°C prepared by mixing 61 ml 0.2 M dibasic sodium phosphate and 39 ml monobasic sodium phosphate.). They were then placed in a decalcifying solution of 10% formic acid in 7% trisodium citrate (7% trisodium citrate solution prepared by

¹ Abbott Laboratories Ltd., Queenborough, Kent.

TABLE 4.1
Light Microscopy

Group	Survival Time	Animal No.
1	3 days	A20, A21
2	5 days	A22, A23, A24
3	7 days	A8, A9
4	10 days	A12, A13
5	14 days	A2, A3, A17
6	21 days	A5, A6, A14
7	28 days	A17, A19, A28
8	35 days	A15
9	42 days	A31, A32
10	84 days	A27, A28

dissolving 70 g in 1 litre of distilled water. 100 ml of 10% formic acid solution were added to 900 ml trisodium citrate solution for 21 days.

After washing under continuous tap water, the mandibles were dehydrated through 10%, 20%, 50% and absolute alcohol, then a further two changes of absolute alcohol. They were then cleaned with two changes of chloroform¹ and finally left overnight in chloroform. The material was then vacuum embedded in three changes of paraffin wax² at 60°C over 6-9 hours and blocked out in fresh paraplast.²

The blocks were cut with Reichert disposable blades mounted on a Spencer AO microtome³ to a thickness of 6 μ . Sections were floated in 1% albumin solution mounted on glass slides and then dried overnight. They were then deparaffinised with 2 x 5 minute changes of xylene⁴, rinsed with absolute alcohol and taken through two baths of absolute alcohol (5 minutes) then rinsed in 50% alcohol (5 minutes) and finally under running water for 5 minutes.

The sections were stained in Weigernt's iron haematoxylin (haematoxylin 1 g produced in 100 ml absolute alcohol; 4 ml of 30% aqueous solution of ferric chloride prepared in 100 ml distilled water and 1 ml hydrochloric acid. Equal parts of both reagents were mixed immediately before use.) for 20 minutes then in 1% acid alcohol (1% hydrochloric acid in 96% alcohol) and blued in Scott's tap water solution (sodium bicarbonate 3.5 g and magnesium sulphate

1 BDH Chemicals Ltd., Poole, Dorset.

2 Oxford Labs, International Co. Sherwood Medical Co., Kildare.

3 American Optical Co., Instrument Division, Buffalo 15, New York.

4 Fisons Ltd., Loughborough, Leicester.

20 g were dissolved in 1 litre of tap water and thymol added as a preservative), then washed under running water. They were then placed in 1% alcoholic eosin (1 g alcoholic eosin¹ in 100 ml of 90% alcohol) for 1 minute, dehydrated in two changes of absolute alcohol, cleared in two changes of xylene and mounted under glass with DPX mountant (BDH Chemicals). Sections were viewed with an Orthoplan Widefield microscope². Selected fields were photographed using Kodak PAN-F film.

b) Collagen Implantation: The Humoral Response

Control of the immune response to collagen requires co-operation between T and B lymphocytes. The only known function of B lymphocytes is to produce antibodies. Antibody-antigen interactions can be measured in many ways. Many tests depend on secondary reactions that follow as a consequence of the primary antigen-antibody interaction. These secondary reactions include precipitation, agglutination and complement fixation reactions. All these methods have been previously used with varied success to demonstrate anti-collagen antibodies.

Alternatively now, radiolabelled, fluorescent or enzyme-coupled antibodies can be used to detect specific molecules in cells or tissues. Specific antibodies in serum to a particular antigen can now be detected and measured by an ELISA.

1 G.T. Gurr, Romford, Essex.

2 E. Leitz (Instruments) Ltd., 48 Park Street, Luton.

Immunoglobulins against implanted bovine collagen were measured using a two-step indirect microplate ELISA using a method similar to that described by Voller et al (1976).

Animals A27 and A32 were used in this study. The dates of sacrifice were 6 weeks (A32) and 12 weeks (A27). For A32 a 5 ml aliquot of venous blood was removed by venepuncture from an ear vein at operation. The blood was allowed to clot and then placed overnight in a refrigerator at +4°C. The following day it was placed in a centrifuge and spun at 2000 revs/min for 10 minutes and the resultant serum was then removed using a suction pipette, placed in a sterile container and then stored in a deep freeze at -40°C until used for the ELISA. Further samples were taken by venepuncture from A32 until the date of sacrifice at 6 weeks. Similar 5 ml samples of blood were taken from animal A27 from week 7 post-surgery with death at week 12. All specimens of serum were stored at -40°C until use and after thawing were kept at +4°C to repeat the experiment.

c) **The Quantitation of Specific Immunoglobulins using an Enzyme Linked Immunosorbant Assay (ELISA)**

Reagents

The coating buffer for sensitising the plates, a carbonate-bicarbonate buffer (pH 9.6) consisted of 1.59 gm of Na_2CO_3 , 2.93 gm of NaHCO_3 and 0.2 gm of NaN_3 made up to 1 litre with distilled water. This was stored at 4°C for no longer than two weeks. The phosphate buffered saline-tween 20 mixture (PBS-tween) consisted of 8 gm of NaCl , 0.2 gm of KH_2PO_4 , 2.9 gm Na_2HPO_4 , $12\text{H}_2\text{O}$, 0.2 gm KCl and 0.5 ml of tween 20 in 1 litre of distilled water. Anti-rabbit

IgG alkaline phosphatase conjugate¹ lot no. 94F 8840 and substrate p-Nitrophenyl phosphate disodium (Sigma No. 104-0) 1 mg/ml in 0.1 M glycine buffer pH 10.4 containing 0.001 M MgCl_2 and 0.001 M ZnCl_2 were used for convenience. Immediately before use, one 5 mg p-nitrophenyl phosphate tablet was dissolved in 20 ml of diethanolamine buffer containing 97 ml of diethanolamine², 800 ml of distilled water, 0.2 gm of NaN_3 and 100 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 1 M HCl was added until the pH reached 9.8 and then distilled water was added to a final volume of one litre.

Method

0.5 ml of collagen paste was shaken with 20 ml of carbonate buffer at pH 9.6 in a sealed container and after thorough shaking a cloudy suspension of collagen was produced. Wells of polystyrene disposable, flat-bottomed, 96 well microtitration plates³ were used on the solid phase. 50 μ l of the collagen suspension was dispersed into each of the wells except for the final row. This now contained carbonate buffer alone to act as a negative control. The plates were sealed and stored at 4°C for 24 hours to allow collagen to adhere to the polystyrene surface. The plates were then washed three times with PBS-tween and tapped dry. 50 μ l test samples of sera were then serially diluted from 1 to 1/960 and dispersed into appropriate wells. A reference serum consisting of pooled sera

1 Sigma Chemical Company, St. Louis, Mo., U.S.A.

2 Fisher Scientific Company, Silver Spring, Md., U.S.A.

3 Flow Laboratories (UK) Ltd., Woodstock Hill, Harefield Road, Rickmansworth, Herts.

from all the samples in serial dilution was used for calibration. All samples and reference sera were diluted in PBS-tween containing 1% newborn calf serum. The plates were sealed and incubated at 4°C for 1 hour. The plates were then washed three times with PBS-tween and tapped dry. 10µl of goat anti-rabbit antibody alkaline phosphatase conjugate (Sigma Chemical Company) was diluted in 5 ml of PBS-tween containing 1% newborn calf serum and 50µl aliquots of the material dispersed into appropriate wells. The plates were again sealed and incubated at 4°C for 1 hour. Plates were again washed three times with PBS-tween and tapped dry before 5-µl of alkaline phosphatase substrate solution was added to each well. Control wells contained neither primary antibody nor alkaline phosphatase substrate. The plates were incubated at room temperature; the rate of substrate degradation was indicated by the colour change which is proportional to the antibody concentration in the sample. The absorbance of the yellow colour produced by the reaction of the enzyme with P-nitrophenyl phosphate was read at 405 nm using a Titertek Multiskan (Flow Laboratories). Sequential readings were taken until optimal curves were obtained. Positive and negative controls were run during the experimentation. The experiment was repeated four times.

d) **Preparation of Undecalcified Sections to Demonstrate
Fluorescent Bone Labelling**

Mandibles of animals given tetracycline and haematoporphyrin bone labels were removed from the deep freeze at -40°C. Before thawing, a section of each mandible containing the operation site was removed by sawing and this block was placed in 4% phosphate

buffered formaldehyde [(BDH Chemicals) (37-40% formaldehyde solution 100 ml, water 900 ml, acid sodium phosphate monohydrate 4.0 g, anhydrous disodium phosphate 6.5 g. The solution has a pH of 7.0.)) for 48 hours at room temperature. After washing, the blocks were dehydrated in alcohols from 10% through 50% to absolute alcohol over 4 hours and then into 100% acetone for 1 hour.

Impregnation of the blocks with resin was produced by placing the block centrally in a mould base and placing Metsee¹ resin S.W. without hardener under vacuum in the mould to the desired level above the block for 6 hours. Part of the resin was removed and replaced by resin mixed with hardener under vacuum for 30 minutes and then the blocks were allowed to polymerise overnight at 37°C. [Metsee mounting plastic consists of polyester resin S.W. (98.75%) and hardener (1.25%). Full hardness is reached 10-15 hours after mixing at 37°C.] The hardened blocks were later mounted on dowel with the same resin. To verify comparable areas from each block were being used, each mounted block was radiographed (Figs. 4a&b) then accurately cut to the level of the second molar tooth before further sections were taken for viewing. Sections approximately 250 μ thick were cut from the second molar area on a Pultra lathe under continuous irrigation.

Resultant sections were initially cemented onto glass slides with a variety of cements for grinding and polishing; none proved suitable to hold the section. The sections were therefore finally hand held before mounting and initially ground on fine emery cloth then polished with Durmax alumina extra-fine polishing fluid (BDH

¹ Metallurgical Services Laboratories, Surrey.

Fig. 4 (a) Undecalcified block (to demonstrate fluorescent bone labelling) showing bony defect and area of second molar tooth before cutting.

(b) Similar block to Fig. 4a after cutting at the level of the second molar tooth in the area of operation. This provides comparable areas for study.

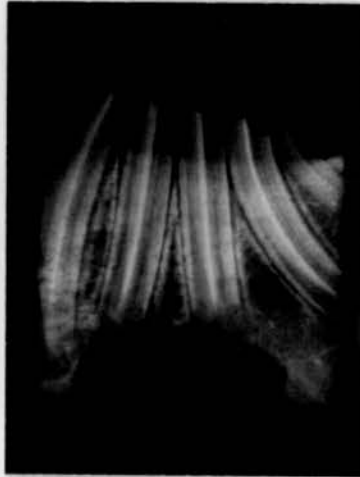


Fig. 4a

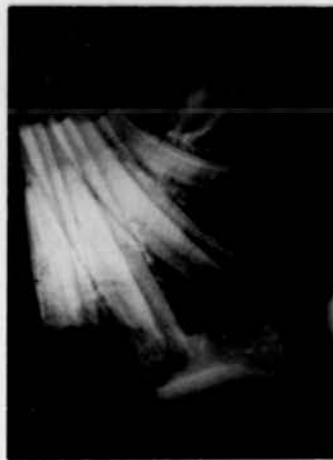


Fig. 4b

Chemicals) on a polishing cloth. Many of the sections disintegrated when ground and polished, but a small number survived and were finally polished to between 100 μ and 150 μ thickness.

The final sections were mounted on glass slides and viewed under a Leitz Orthoplan universal large field microscope with U.V. excitation. The ultra-violet source was a mercury lamp HBD 200W/4 with filter D in position 2. Selected specimens were photographed with a photomicrographic camera (Leitz) using Agfacolor professional film XRS 100.

e) **Cytotoxic Effects of Heterologous Collagen on a Culture of Human Oral Fibroblasts**

Materials which are biocompatible should not interfere with the growth and maturation of cell populations in close proximity. To assess the cytotoxicity of any material for human use, its effect on a population of human cells should be elicited. The growth of a colony of human fibroblasts can be followed and any adverse effects can be seen at a cell or subcellular level.

The culture medium for this investigation was made up as follows:-

Glasgow's minimal essential medium¹ 10%; Sodium pyruvate¹ 1 m Mol/litre; L-glutamine¹ 200 m Mol/litre; Non-essential amino acids¹ 1%; Pooled human serum 10%; Sodium bicarbonate¹ 20 m Mol/litre; Penicillin¹ 100u/ml; Streptomycin¹ 100 μ g/ml; Fungizone¹ 0.25 μ g/ml.

¹ Flow Laboratories Ltd., Woodcock Hill Industrial Estate, Harefield Road, Rickmansworth.

The culture medium was buffered with sodium bicarbonate and the pH maintained by incubation in an atmosphere of 95% air and 5% CO₂. Antibiotics and antifungal agent, Fungizone, were added to prevent infection. Pooled human serum was prepared by clotting whole blood obtained by venepuncture at 4°C overnight then centrifuging the blood at 2000 rpm for 10 minutes and removing the serum supernatant. The serum was heat inactivated by placing it in a 60°C oven for 30 minutes. Sera were sterile filtered and stored in 5 ml aliquots at -20°C.

Method

Pieces of normal human oral mucosa obtained from healthy subjects after the surgical extraction of teeth were sent to the laboratory in transport medium. This medium consisted of Glasgow's M.E.M. 10; sodium pyruvate 1 m Mol/litre; L-glutamine 200 m Mol/litre, non-essential amino acids 1% and newborn calf serum 10%.

In the laboratory, the mucosa was cut into fragments of approximately 1 mm³. Eight fragments were placed in each petri dish and were anchored in place using a sterile glass coverslip. Tissues were bathed in the culture medium, 4 ml per dish and incubated in 95% air 5% CO₂ being left undisturbed for two days. Outgrowths from the explants were monitored by phase contrast microscopy. As the cultures reached confluence, they were washed in 10 ml of phosphate buffered saline and 1 ml of trypsin solution, (a solution of trypsin 0.05% and chicken serum 0.05%) made up in sterile phosphate buffered saline (PBS), and disodium ethylene diamine tetra-acetic acid (EDTA) 1 m Mol/litre was added to each dish which was then incubated at 37°C until cells began to detach.

The cells were gently dislodged by pipetting and medium containing serum was added to stop further trypsinization. The cell suspension was then centrifuged at 500rpm for 5 minutes and the supernatant decanted. Fresh medium was used to resuspend the cells which were plated in a 1:2 split ratio. Small pieces of collagen were placed in the centre of a confluent layer of fibroblasts and the cells were monitored after 24 hours and 48 hours and their condition noted. The condition of cells adjacent to collagen was viewed by phase contrast microscopy (Leitz) and electron microscopy¹ to show the effects at cellular and subcellular level.

f) Preparation of Cultures for Electron Microscopy

Method

The growth medium was removed from the petri dishes containing the fibroblast/collagen culture and replaced with 2.5% glutaraldehyde in phosphate buffer at pH 7.2. This phosphate buffer was prepared as follows:-

A 0.2 M solution of dibasic sodium phosphate $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ was prepared using 71.64 g with distilled water to 1 litre. A 0.2 M solution of monobasic sodium phosphate $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ was prepared using 31.21 g with distilled water to 1 litre. A 0.2 M phosphate buffer at pH 7.2 at 25°C was prepared by mixing 72 ml of 0.2 M dibasic sodium phosphate and 28 ml of 0.2 M monobasic sodium phosphate. The cells were fixed overnight at 4°C.

¹ Pye Unicam Ltd., York Street, Cambridge.

An ampoule containing solid osmium tetroxide was dropped into a clean brown glass stoppered bottle and phosphate buffer added to produce a 1% solution. The cells were then post-fixed in the 1% osmium tetroxide in phosphate buffer at pH 7.2 for 1 hour. After osmication the culture was washed in 10% ethanol and the cells dehydrated in absolute alcohol with three changes at intervals of 30 minutes. Cells were then cleared in propylene oxide for 30 minutes.

Araldite™ mixture was prepared in the following way:-

500 ml Araldite™ resin (CY212) was thoroughly mixed with 500 ml hardener (HY 906) at room temperature. 5 ml of accelerator (DMP 30) were mixed with 20 ml dibutyl phthalate and kept at 4°C. 1 ml of accelerator mixture was added to 19 ml of resin mixture and mixed by continuous rotation for at least 12 hours at room temperature.

The cultures were transferred from propylene oxide to shallow polythene containers filled to a depth less than 1/4" of Araldite™ mixture and left at room temperature for at least 12 hours. The polythene containers were then placed in an oven at 60°C, the Araldite™ decanted and replaced by fresh warmed Araldite™ and hardened by replacement in an oven at 60°C for 48 hours.

The Araldite™ blocks were mounted on dowel with sealing wax and thin sections obtained from the blocks. The sections were mounted on Gilder grids¹ without a supporting membrane.

¹ Polaron Equipment Ltd., Watford.

The sections were stained by floating the grids, section down, on prepared lead citrate solution 0.2% in 0.1 N NaOH¹ for 2 minutes, then passing the grids held in forceps under a solution of distilled water for 10 seconds. The grids were then immersed in 5 ml of a saturated solution of uranyl acetate in 50% ethanol¹ prepared immediately before use, for 10 minutes and finally held in forceps moved under a 50% ethanol solution for 20 seconds. Sections were examined using a Phillips EM301 (Pye Unicam Ltd.).

¹ TAAB Laboratories, Emmergreen, Reading.

CHAPTER 5

CLINICAL METHODS II. HUMAN IMPLANTATION STUDY

Introduction

A bony cavity in the mandible or the maxilla from which pathological tissue has been removed begins healing with the organisation of the extravasated clot. Blood clot is replaced by osteogenic granulation tissue. Osteogenesis spreads inwards from the periphery and in a small defect the blood clot is replaced by bony trabeculae within a few weeks. The healing process is completed by cells that differentiate from the endosteum or marrow spaces with little or no part being played by periosteum (Kramer et al 1968). The defect fills from the periphery over a period of many months. Incomplete bony regeneration following the removal of a maxillary cystic area may be encountered and there are instances when such cavities never fill with bone, only scar tissue. In others, the retained radiolucent area does reduce in size, but incomplete bony infilling remains for many years.

Radiographic studies have shown that most of the bony healing will occur in the first post-operative year with little further radiographic change seen after four years (Rud et al 1972). Experiments in the dog confirmed that when both the inner and outer cortical plates are destroyed, the cavity is likely to fill only with fibrous tissue (Hjorting-Hanson & Andreason 1971).

In the maxilla, there is little cancellous bone that can contribute to endosteal osteogenesis and the likelihood of total osseous repair is further reduced in large cavities, especially when both cortical plates are penetrated. Healing of bony cavities in

the jaws, shown radiographically, will be used to show the effect of implanting the collagen paste in man and a comparison made with similar non-implanted areas used as controls for this.

Patient Selection

One hundred patients (41 males and 59 females) were included in this study. All patients presented to the Edinburgh Dental Hospital and School with an acute or chronic inflammatory area in the mandible or maxilla. A clinical history was obtained from each patient and if there was a history of an auto-immune disease, a collagen related disorder or a current pregnancy, they were excluded from the trial. All patients had radiographs taken of the affected area in the Radiology Department of the Edinburgh Dental Hospital and School. Patients presenting with acute symptoms had abscesses drained when present or were placed on a five day course of antibiotic if a cellulitis was present clinically. The antibiotics used were phenoxymethyl penicillin 250 mg q.i.d. or erythromycin 250 mg q.i.d. if the patient claimed hypersensitivity to penicillin. All patients were operated on after an acute exacerbation had subsided.

A schedule for 100 patients had been previously compiled by a random selection method; each patient was allocated a consecutive number and the appropriate treatment given to that specific number. In all cases the area of chronic infection or a cystic lesion in the mandible or maxilla was to be explored and all pathological tissue removed by curettage. From the compiled schedule, 50 of the patients would have the area curetted free of the pathological tissue and the area then allowed to fill with blood clot. The

other 50 patients had the affected area, after curettage, filled with the experimental collagen paste.

From the schedule of the 50 patients placed in the control group, 22 were male and 28 female. The collagen implant group consisted of 19 males and 31 females.

The trial submission was approved by the local Ethical Committee.

Method

All patients were treated under regional block or local infiltration anaesthesia. A suitable flap of mucoperiosteum was raised widely over the pathological area so that the resultant defect would be totally covered by soft tissue when the flap was replaced. If cortical bone had not been breached, a window was made with a chisel or conventional surgical burrs under warm saline irrigation to allow good access to the pathological cavity. When the apices of teeth were involved in the pathological lesions, and these teeth were non-vital, then an apical seal of dental amalgam was placed in the root apex if the conventional root filling did not adequately seal the root canal. The cavity was thoroughly curetted of all pathological debris and irrigated continuously with warmed normal saline. When the cavity was deemed clinically to be clear of all debris, it was dried with sterile pledgets and in cases selected for implantation, the cavity was completely filled with the experimental collagen paste dispensed from its sterile syringe. In the control group the cavity was allowed to fill with blood clot. The mucoperiosteal flap was sutured in its original position with 3/0 Dexon™ sutures.

All patients were asked to return immediately if there was persistent pain, swelling or erythema or if they became generally unwell. Antibiotics were not prescribed routinely at the time of operation. Only patients who returned with cellulitis were given penicillin or erythromycin in appropriate dosage.

After an initial post-operative review at two weeks, all patients were asked to return at three months and subsequently at six months and 12 months post-surgery for clinical assessment and radiographs to be taken of the operated area.

Radiology

The majority of bony lesions operated on were in the upper anterior region of the maxilla (75) and lower anterior region of the mandible (19). Six were sited more posteriorly in both jaws.

In the anterior regions of mandible and maxilla, intra-oral dental film (Kodak Ultraspeed D) was used when radiographs of small lucent areas were taken and where the pathological areas were more extensive then occlusal film (Kodak Ultraspeed D) was used.

All radiographs were taken with a Phillips Oralix machine. Intra-oral films were taken from a fixed focal distance (FFD) of 16 cm with a tube angle of 55° . The exposure time for the upper anterior region was 0.35 secs and for the lower anterior region of 0.2 secs. Anterior occlusal films were taken at 55° but with an FFD of 18 cm and an exposure time of 0.56 seconds.

Radiolucent areas in the posterior areas of the mandible and maxilla were demonstrated and compared by an orthopantomograph

(OPG). Radiographs were taken on the Morita Panex machine¹; the machine was constantly set so all films were comparable. The relevant section on the OPG was removed for comparison.

All films were processed automatically in a Pantomat (Siemens Ltd.) using M & B Matalex developer and M & B fixer plus. Oral and occlusal films were processed on a six minute cycle and the OPG on a four minute cycle. Films were dated and clearly labelled with patients' names and the date.

When patients returned at three months, six months and one year, similar radiographs to those taken initially were obtained so a direct comparison could be made of the change in radiodensity of the operated area. For a more reliable comparison of operated areas, the lucent zone on the radiograph was measured in two directions at right angles, with an allowance for magnification, and three groups were then compiled. The groups consisted of lesions less than 1 cm in diameter; those between 1 and 2 cm and those greater than 2 cm. It was considered the results would be of more value if areas of similar dimensions within the jaws were compared. Bony defects less than 1 cm in diameter were called 'small' (S), between 1 and 2 cm 'medium' (M) and those greater than 2 cm were termed 'large' (L).

In the collagen implanted group, 27 defects were small, 18 medium and 5 large. By comparison in the implanted group, 27 were small, 19 medium and 4 large. In the implanted group, 37 lesions were in the maxilla and 13 in the mandible and in the control group 38 in the maxilla and 12 in the mandible (Tables 5.1 and 5.2).

¹ Morita Corporation, Japan.

TABLE 5.1

CONTROL GROUP

	Site	Size of Bony Defect			Total
		S <1 cm	M 1-2 cm	L >2 cm	
Male	Maxilla	12	5	0	17
	Mandible	2	2	1	5
Female	Maxilla	11	8	2	21
	Mandible	2	4	1	7
Total		27	19	4	50

TABLE 5.2

COLLAGEN IMPLANT GROUP

	Site	Size of Bony Defect			Total
		S <1 cm	M 1-2 cm	L >2 cm	
Male	Maxilla	9	3	1	13
	Mandible	0	4	2	6
Female	Maxilla	16	6	2	24
	Mandible	2	5	0	7
Total		27	18	5	50

CHAPTER 6

RESULTS

This study has investigated the use of a formulated collagen as a bone implant in several ways. Materials which are biocompatible would be expected to behave differently from non-biological implants which may be regarded by the host as foreign. The bio-compatibility of the test material has been shown in vitro by the effect on human fibroblast tissue cultures. The effect of the material on the stages of wound healing has been demonstrated in vivo using comparable bony lesions in the rabbit mandible as test and control sites. A longitudinal histological study of the bony wounds from three days post-operatively until 12 weeks has shown the progress of test and control sites. Paralleled with the histological findings, early mineralisation and subsequent patterns of calcification have been compared by bone labelling. It was also considered that differences in the vascularity of the operated areas would be informative.

All foreign proteins elicit an antibody response; cells present on the histological study of healing demonstrate the type of reaction elicited, but more specifically antibody levels against the implant material have been measured. Radiology of the rabbit mandibles and the above results provide the basis for interpreting the findings of the human study.

The results are presented in the following order:-

1. Cytotoxic effects on fibroblast culture.
2. Operative surgery, histology and bone staining on the animal models.

3. Antibody response.
4. Radiology of the animal model.
5. Human implantation study.

The relevant figures and tables are shown at the end of each of these sections.

1. Cytotoxic Effect of Collagen on Oral Fibroblast Culture

Materials which are biocompatible should have no cytotoxic effects on cells grown in tissue culture. Twelve different human fibroblast cultures were grown from oral mucosa as previously described. Pieces of collagen were placed in the centre of confluent layers of cells and the effect viewed after 24 and 48 hours. The relationship of human oral fibroblasts grown in tissue culture with collagen implant material is shown in Figs. 5 to 12. At low magnification (x 320), a normal growing and dividing fibroblast colony is shown to be closely related to pieces of the collagen matrix (Fig. 5). At higher magnification (x 640) under phase contrast microscopy (Figs. 6 & 7), the cells do not appear to be damaged by the presence of the material and would appear to have an intimate relationship lying inferiorly, superiorly or possibly growing through the matrix. During processing it may be expected that the material would be lost but it would seem that the fibroblasts may actually hold the implant in situ.

At higher magnification (x 2,100) electron micrographs further demonstrate this very close relationship between cell and implant (Fig. 8). The fibroblast nuclei were of normal size and shape with normal numbers of compact nucleoli, no irregularities and no intranuclear inclusions. An indentation is shown in Fig. 8 but

this was not typical. The cell cytoplasm appeared normal and contained a satisfactory distribution of organelles. The mitochondria appeared well preserved but typical of cells in culture there was a poor complement of endoplasmic reticulum but an abundance of free polyribosomes reflecting active protein synthesis.

Golgi complexes were poorly developed probably reflecting the emphasis on growth and multiplication rather than differentiation and function. Some empty spaces with no apparent membrane structures were seen; these were thought to represent a fixation artefact, possibly the result of loss of lipid material.

At magnifications of x 5,600 and x 14,000 (Figs. 9 & 10) the intimate relationship between cell wall and implant fibrils is further demonstrated, several areas of direct contact being seen. The results are confirmed by Fig. 11 again showing collagen fibrils adjacent to the cytoplasmic membrane and at higher magnification (x 14,000 Fig. 12) the cell wall appears to hold the fibrils in situ. Cells in culture grew and matured without effect from the exogenous implant material and this was verified by a dye exclusion technique using trypan blue.

Fig. 5 Normal growing and dividing fibroblasts in close relation to collagen implant material.

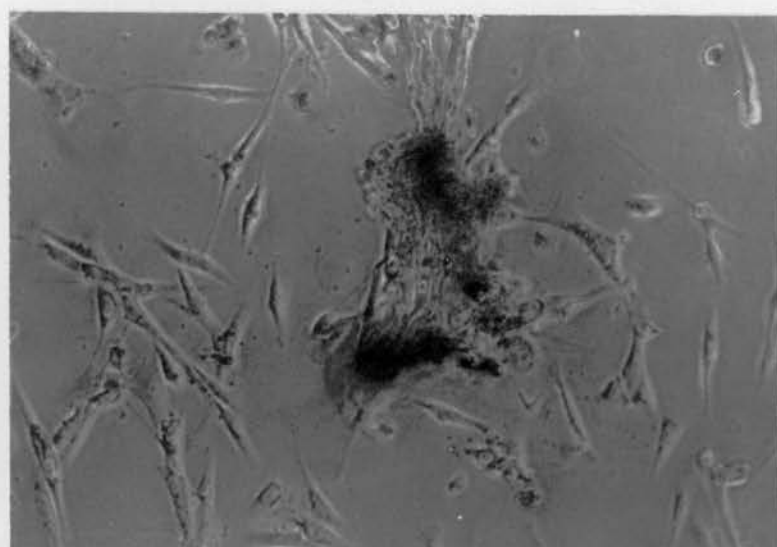
Phase contrast x 320

Fig. 6 Higher power view of implant material seen in Fig. 5 with fibroblasts appearing to lie inferiorly, superiorly, or through the matrix.

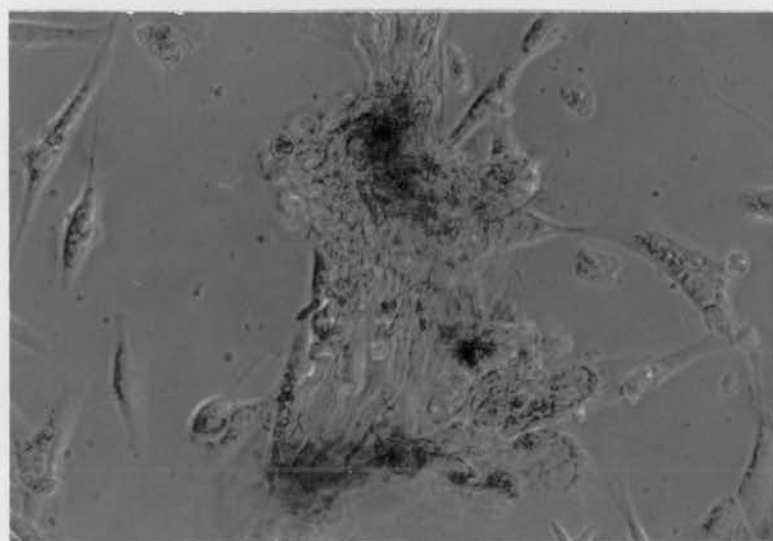
Phase contrast x 640

Fig. 7 An area adjacent to Fig. 6 confirming the intimate relationship shown in Fig. 2.

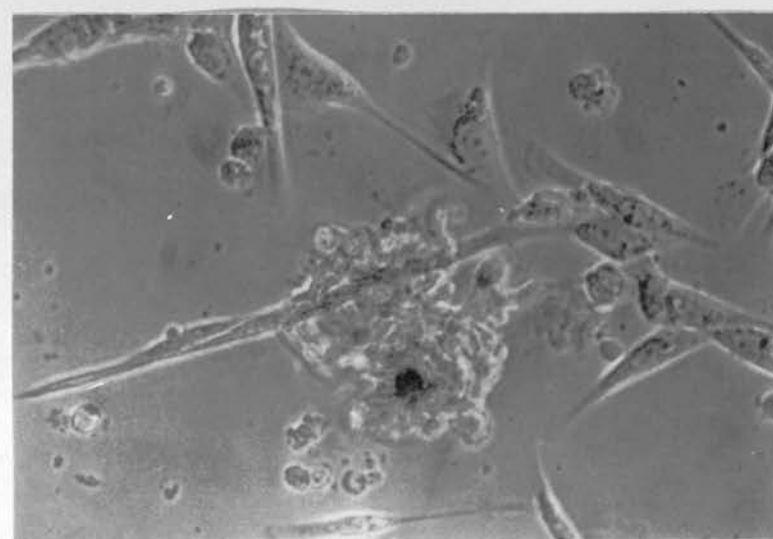
Phase contrast x 640



5



6



7

Fig. 8 Low magnification micrograph shows the characteristic features of massed collagen fibres seen lying in different orientations and closely related to a fibroblast. The nuclear size and shape are normal but an indentation is noted. There is a normal distribution of chromatin. The cytoplasm appears normal showing rough endoplasmic reticulum, mitochondria, electron dense granules and empty spaces with no membranes, probably a fixation artefact, possibly due to loss of lipid material.

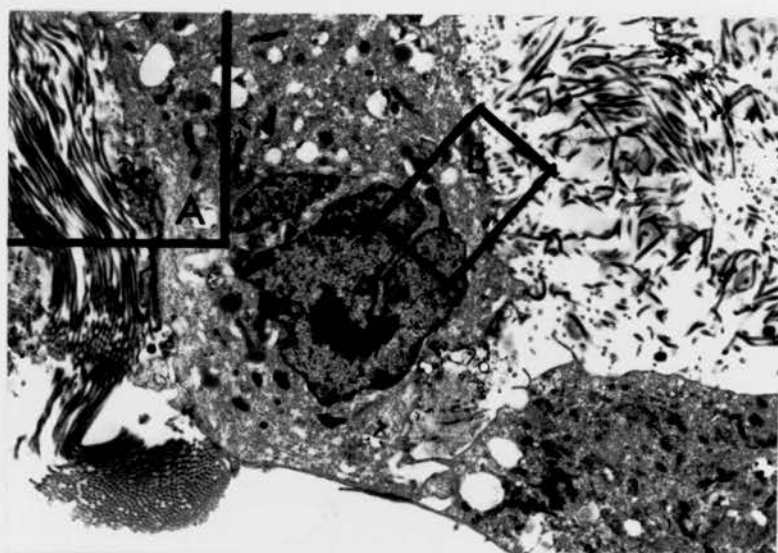
Electron micrograph x 2,100

Fig. 9 Higher power view of area A confirming very close relationship of fibroblasts with collagen implant and confirming intact plasma membrane.

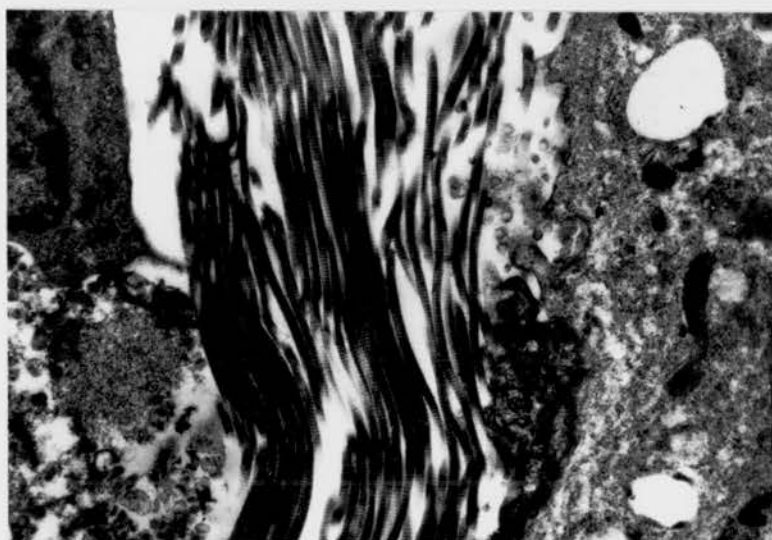
Electron micrograph x 5,600

Fig. 10 Higher power view of area B showing nucleus and nuclear membrane, mitochondrion and several areas of intimate contact of cellular membrane and collagen. Collagen fibrils show characteristic cross-bonding.

Electron micrograph x 14,000

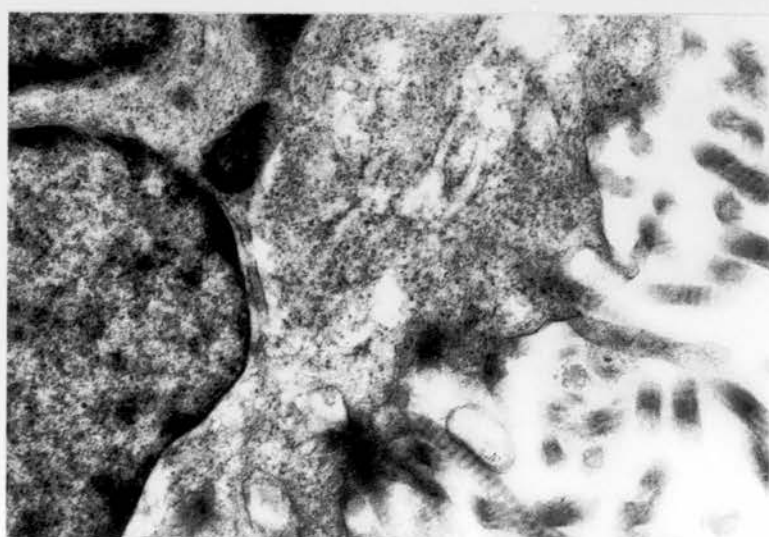


8



AREA
A

9



AREA
B

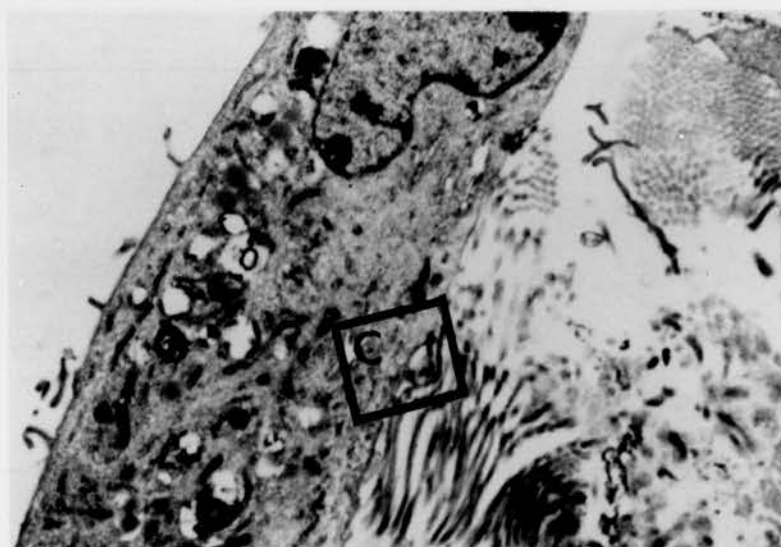
10

Fig. 11 Close relationship of normal fibroblast and collagen fibrils.

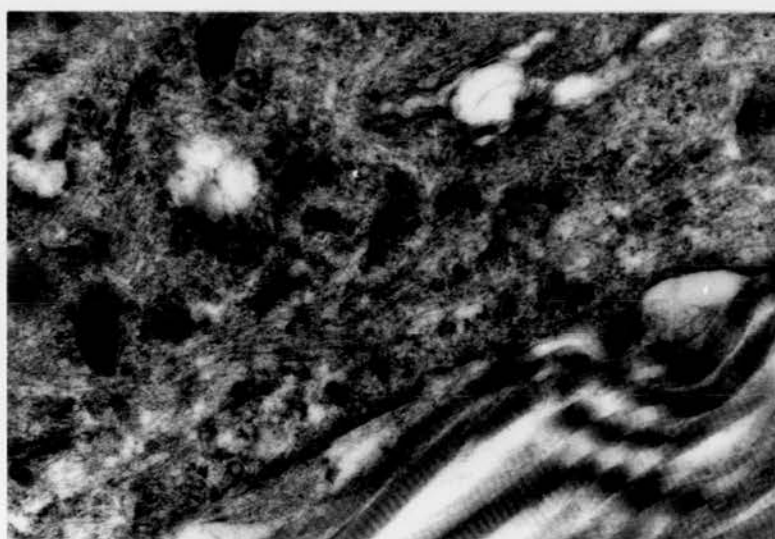
Electron micrographs x 3,500

Fig. 12 Higher power view of area C which shows the cell appears to hold the collagen fibrils in situ.

Electron micrograph x 14,000



11



12

2. Operative Surgery using the Animal Model

Thirty one rabbits were finally operated on in this study, one died immediately post-operatively leaving 10 groups of three rabbits per group. The animals were operated on in the order of A1 to A32 because of the lack of accommodation (only six rabbits could be housed at any time); the survival times and group numbers do not follow in a chronological order. The experimental date concerning each animal used in the studies is summarised in Table 6.1.

Animal A11 died under anaesthesia, probably due to the anaesthetic agents used. As a result Group 4 (10 day survival) then had animal A13 substituted for A11. To compensate for this loss, as the rabbits with a three day survival were to be used for the longitudinal histological study alone, only two animals were placed in this group, A20 and A21. In Group 10 (84 day survival), animal A26 died immediately postoperatively, again probably due to the anaesthetic agents. A28 was placed in this group which then consisted of A25, A27 and A28. Animal A29 injured itself in its cage and was therefore felt to be unsuitable for use. Finally, Group 9, the last group operated on therefore comprised animals designated A30, A31 and A32.

Wound infection was seen in only two animals. One, A20 was sacrificed at three days and therefore not treated; the other, A3, was successfully treated by local cleansing and a topical poly-antibiotic medicament.

Two animals died prematurely, A17 (Group 7, 28 day survival) died at 14 days and A28 (Group 10, 84 day survival) died after four weeks. The cause in both cases was respiratory infection.

TABLE 6.1

ANIMAL ALLOCATION AND PROGRESS

Group/ Survival	Animal No.	Light Microscopy	Fluorescent Microscopy	Radiology	Immunology	Comments
(5) 14 days	A1		+			
	A2	+		+		Used for microcirculation. Wound infection.
	A3	+		+		
(6) 21 days	A4		+			
	A5	+		+		
	A6	+		+		
(3) 7 days	A7		+			
	A8	+		+		
	A9	+		+		
(4) 10 days	A10		+			Died under anaesthesia.
	A11					
	A12	+		+		
	A13	+		+		
(8) 35 days	A14		+			Adverse reaction to haemato- porphyrin. Killed at 21 days.
	A15	+		+		
	A16		+			
(7) 28 days	A17	+		+		Died at 14 days.
	A18		+			
	A19	+		+		
(1) 3 days	A20	+		+		Wound infection.
	A21	+		+		
(2) 5 days	A22	+		+		
	A23	+		+		
	A24	+		+		
(10) 84 days	A25		+			Died immediately post-operation.
	A26					
	A27	+		+	+	Died after 28 days.
	A28	+				
	A29					Unsuitable for use on delivery.
(9) 42 days	A30		+			
	A31	+				
	A32	+		+	+	

Rabbit A14 suffered a toxic reaction to haematoporphyrin. After the subcutaneous injection of the dye at day 14, the animal began to lose muscle bulk, developed joint swelling and ataxia. The condition steadily deteriorated over the following week and it was then sacrificed at 21 days.

In view of this toxic reaction to haematoporphyrin, it was decided it should not be given to animals A25 and A30 in Group 9 and 10 (42 and 84 day survival). The final schedule of administration therefore labelled these animals only with tetracycline and it was given at intervals of two weeks until sacrifice.

All rabbits showed a weight loss immediately post-surgery of up to 200 gm, but the pre-operative weight was normally regained by the first post-operative week, although three animals did require two weeks.

a) Microangiographic study on the new vascular circulation in the healing bony defect

It was hoped that this study would demonstrate differences in the early phase of wound healing between control and test sites.

Animal A2 (Group 5, 14 day survival) was re-anaesthetised with Hypnorm™ intra-muscularly and Diapezam™ intra-venously as previously described (0.2mg/Kg body weight of Hypnorm™ intra-muscularly and then 1mg/Kg Diazepam™ intra-venously). The infusion of Hypaque™ saline was then begun. Within the first 5 minutes, the animal became tachypnoeic then the rate of respiration slowed and death due to cardio-respiratory arrest was noted 10-15 minutes after the infusion began. As the infusion proceeded over the following two

hours, there was marked soft tissue oedema in the head and neck region with sero-sanguinous exudation appearing at the external nares. A total volume of 700 ml of radiopaque fluid was infused into the animal.

Radiographs of the bisected mandibles after removal of the surrounding soft tissues, described in Chapter 3, failed to demonstrate any radiopaque dye in either the implanted or control operation areas. There was no difference noted between each half of the mandible radiographically. Thus in view of the difficulty of the technique and in the absence of a demonstrable result, this method of assessment of healing was abandoned.

b) Light microscopy

A longitudinal histological study was used to show the response of the tissues to implanted collagen; the effects on stages of wound healing; the disposal of the implant material and its effect on the inflammatory response.

i) 5 days post-operatively

Animals A22 and A23 were sacrificed at 5 days. On the implanted side (right) much of the collagen implant material was seen in situ. The interstitial areas between the material showed abundant granulation tissue (Figs. 13a,b,c). Small fragments of bone remaining from the operative procedure were seen to be resorbed by local osteoclasts and some trabeculae of woven bone were noted within the rich granulation tissue.

The control lesion on the left side (Figs. 14a,b) demonstrated very similar features with abundant granulation tissue, some areas of residual bone fragments and the formation of areas of woven

bone. The micrographs of the granulation tissue (x 360) from implanted and control areas (Figs. 13c & 14b) showed no significant differences. Neither demonstrated the retention of the features of the acute inflammatory response due to infection nor a foreign body giant cell reaction in relation to the implanted area.

ii) 10 days post-operatively

The animals sacrificed at 10 days were A12 and A13. At low magnification (x 40) the implanted area (right) demonstrated strands of cell rich fibrous tissue (Fig. 15a) which were maturing from the granulation tissue. Much implant material was still present. At higher magnification x 360 (Fig. 15b) fibrous tissue was noted to be developing adjacent to the implant material; again no foreign body giant cells were noted in relation to the foreign matrix. In contrast the control area (left) (Fig. 16a) demonstrated a more advanced state of healing with finely trabeculated bone advancing into the fast maturing fibrous tissue (Fig. 16b).

iii) 14 days post-operatively

Animals A2 and A3 were sacrificed at 14 days. Collagen implant material was still demonstrable at 14 days on the right side although it was beginning to fragment and the operation site was being replaced by mature granulation tissue (Fig. 17a). The healing process appeared to be maturing with early fibrocytes seen swarming over and through the fragmenting implant material (Fig. 17b). Although at this stage the material was becoming fragmented the debris was not being removed by giant cells. The control area (Fig. 18) showed a very mature fibrous tissue and advancing front

of bone of repair.

iv) 21 days post-operatively

The animals sacrificed at 21 days were A5 and A6. By 21 days the implanted area showed an advancing bony front with progression to lamellar bone (Fig. 19). Implant material was however still visible and seemed to be occluding the surgical defect thus slowing the advancing reparative bone. The control lesion (Fig. 20), by comparison, showed the surgical defect to be almost completely occluded by mature granulation tissue and plentiful trabeculae of bone.

v) 28 days post-operatively

Animals A17 and A19 were sacrificed at 28 days. The surgical defect on the implanted side was still present and advancing tissue of repair had not as yet filled the area (Fig. 23). By comparison at 28 days on the control side, trabeculated bone was seen to extend throughout the surgical defect (Fig. 24).

vi) 35 days post-operatively

The animal sacrificed at 35 days was A15. At this time, no evidence of any retained implant material was found. There was continued formation of woven bone within the fibrous granulation tissue which was seen to be advancing throughout the whole of the implanted surgical defect (Fig. 27).

The control area by comparison demonstrated complete bony replacement of the surgical defect at 35 days (Fig. 28).

vii) 84 days post-operatively

Animal A27 was sacrificed at 84 days. A comparable histological indication of healing to that shown at 35 days on the control side was not seen on the implanted side until 84 days. At this time, the implanted surgical defect was replaced by bone showing a comparable trabecular pattern (Fig. 31).

c) Fluorescent bone labelling

The effect the collagen implant had on calcification of the surgical defect was shown directly by an in vivo bone staining technique using tetracycline and haematoporphyrin.

Animal A4 was sacrificed at 21 days. The control area (left) at 21 days showed an ordered trabecular network of bone which was seen to be extending from the periphery of the control lesion towards its centre (Figs. 21a,b,c). Bone labelling showed an uptake of three labels used in this animal. The tetracycline given on the day of operation showed a continuous narrow yellow band peripherally closely related to the periosteum. The second wider band of haematoporphyrin (orange) given at seven days, showed a uniformly advancing front indicating a more pronounced formation of new bone. The second tetracycline label (yellow) given at 14 days showed a wider zone growing into the defect. The pattern of growth demonstrated showed parallel trabeculation from the periphery to the centre of the lesion. The banding would indicate little bone formation before day seven and then a marked increase in the deposition of new bone from day seven to 14 which was maintained or increased over the following seven days. The speckled areas noted, were a constant feature and difficult to interpret as they only

showed yellow staining. They were thought to represent contained osteocytes and thus appeared throughout the stained areas. Higher power views (x 560) showed the incremental pattern of ossification over the 21 days (Fig. 21c).

In comparison (Fig. 22) at the site of collagen implantation the advancing bony front was much less well orientated, lacked the fine trabeculation seen in Figs. 21a and 21b and was noted to be restrained when coming into contact with the non-staining, amorphous implant material. Contained osteocytes were seen but they were sparse in comparison with the control area. Isolated lacunae however (Fig. 22c) showed a very similar incremental pattern of ossification as the control (Fig. 21c) which would confirm that the underlying healing process had not been altered fundamentally, only retarded and disorientated.

At 28 days (Figs. 25a&b), animal A18 was sacrificed and in this animal only tetracycline was used as a bone marker. There was continued regular organised ossification occluding the control surgical defect with a high concentration of contained osteocytes that seemed to make a concentric pattern around the lacunae (Fig. 25b). When comparing the implanted area (Fig. 26), the presence of foreign material seemed to retard the healing and the pattern for ossification seemed to show a much more disorganised and rather patchy distribution where it was laid down around retained implant matrix.

At 35 days (Animal A16) when the implanted defect was compared to the control area (Fig. 29a), the areas of ossification in relation to the implant were still more disorganised and there were many more localised areas of ossification. (An example is shown in

Fig. 29b.) The trabeculation pattern of the newly formed bone was noted to be sparse and less well organised. The labelled control lesion (Fig. 30) demonstrated at 35 days confirmed the histological findings that the surgical defect was fully replaced by a calcified matrix which showed a much more regular pattern throughout.

It was finally confirmed that by 84 days in animal A2 the implanted area was totally replaced by bone (Fig. 31). As tetracycline only was used as a bone marker, the pattern was not well defined but the stain was shown to be throughout the original surgical defect. Finally there was therefore little difference between the bone patterns shown on the implanted and control areas but the time for healing was delayed considerably by the implanted collagen.

Fig. 13 5 days - collagen implant

(a) Section shows implanted material (M) associated with abundant granulation tissue (G) and spicules of vital bone (B). Trabeculae of woven bone (W) are identified.

H & E x 40

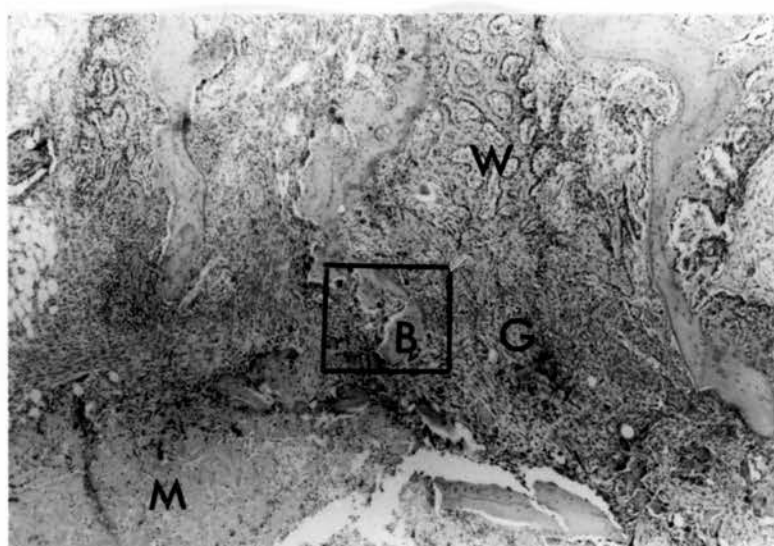
(b) Section shows resorption of vital mature bone by osteoclasts (arrowed) and development of woven bone within the granulation tissue.

H & E x 144

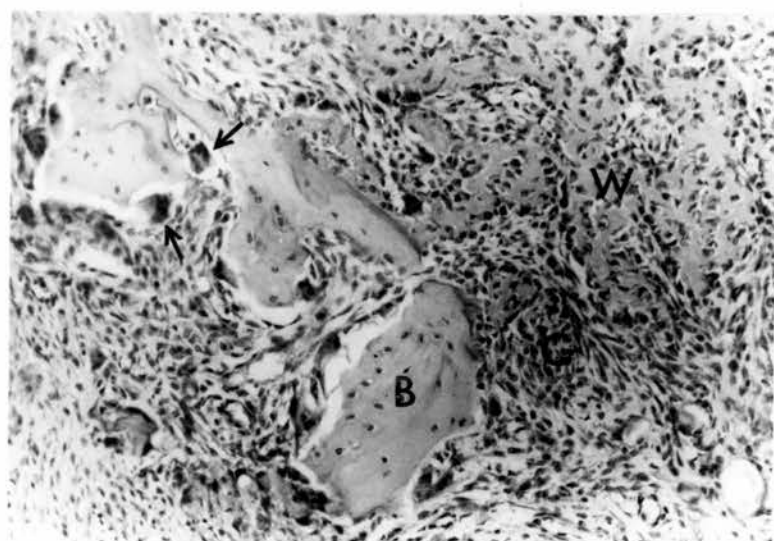
(c) Fibroblast rich granulation tissue.

H & E x 360

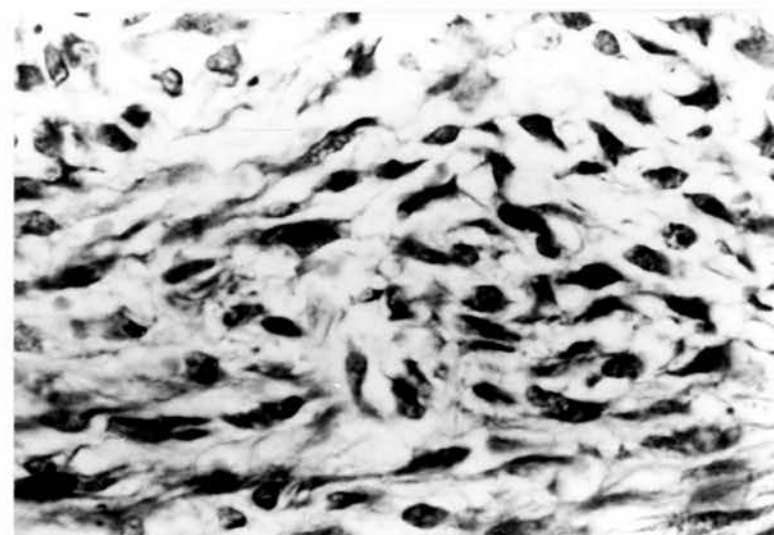
Fig. 13



(a)



(b)



(c)

Fig. 14 5 days - control

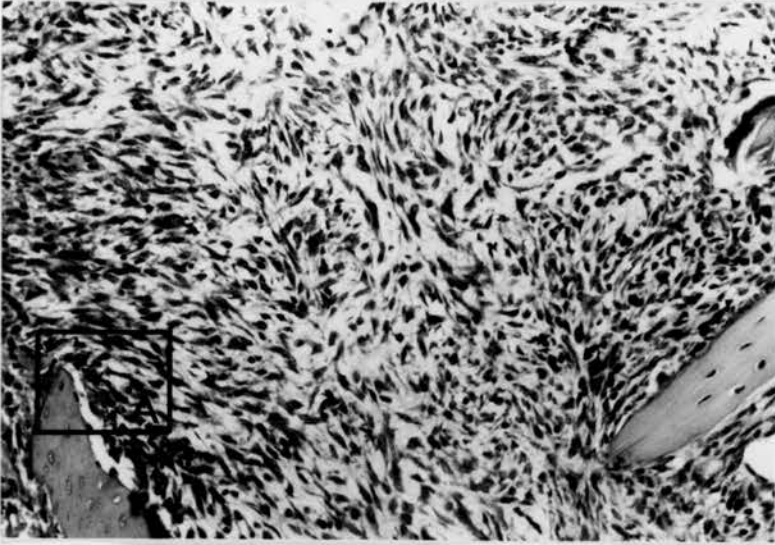
(a) Comparable field with Fig. 16 shows cytologically similar granulation and bone tissue.

H & E x 144

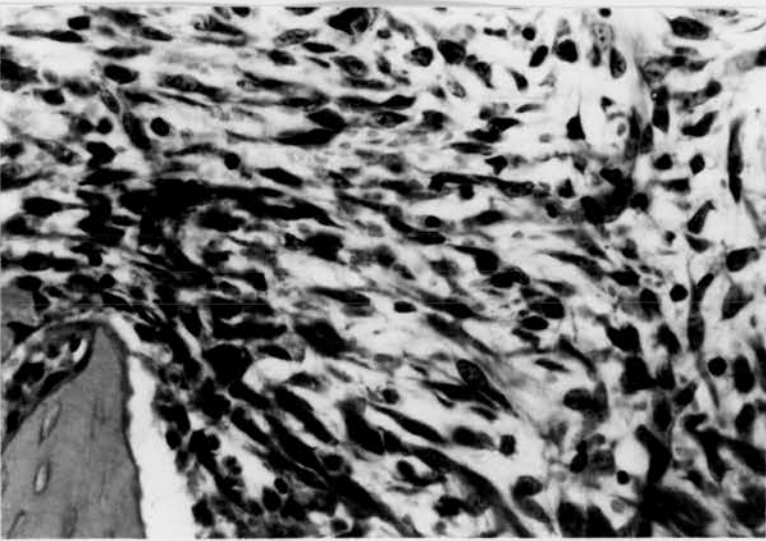
(b) High power view of area A in Fig. 14a showing granulation tissue with no significant difference from that seen in Fig. 13c.

H & E x 300

Fig. 14



(a)



(b)

Fig. 15 10 days - collagen implant

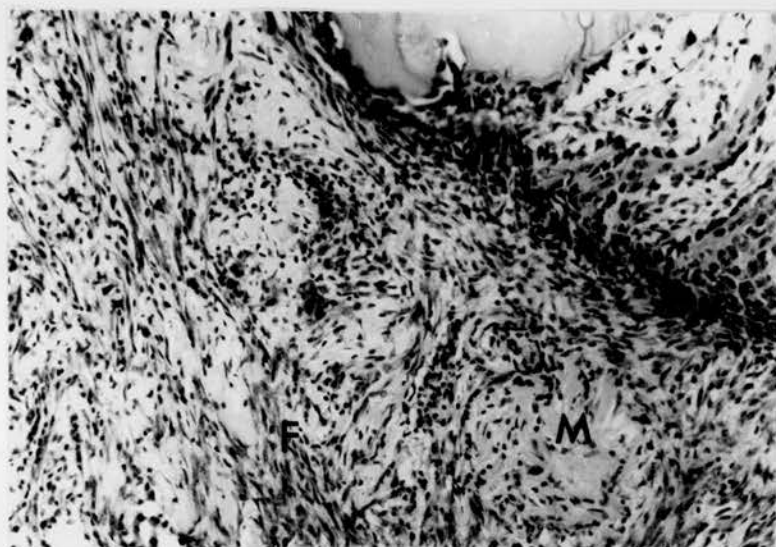
(a) Strands of cell rich fibrous tissue (F) maturing from granulation tissue associated with implant material (M).

H & E x 40

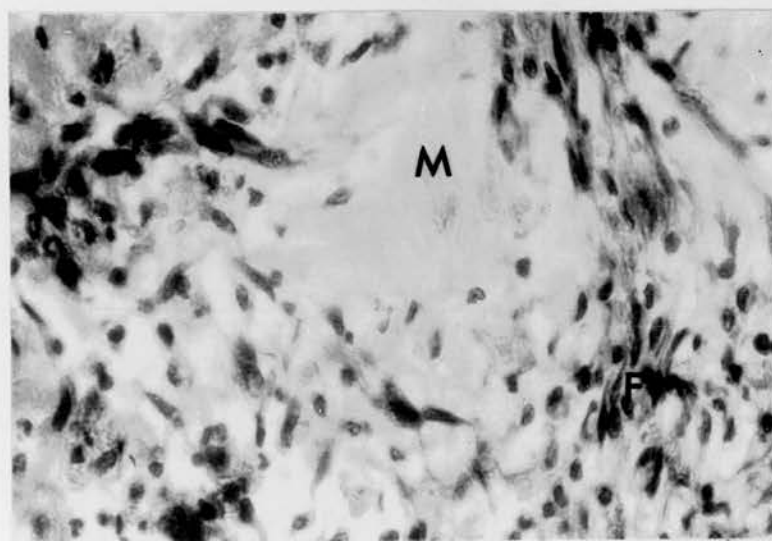
(b) Higher power view of fibrous tissue (F) developing around implant material (M). Note absence of inflammatory cells.

H & E x 360

Fig. 15



(a)



(b)

Fig. 16 **10 days - control**

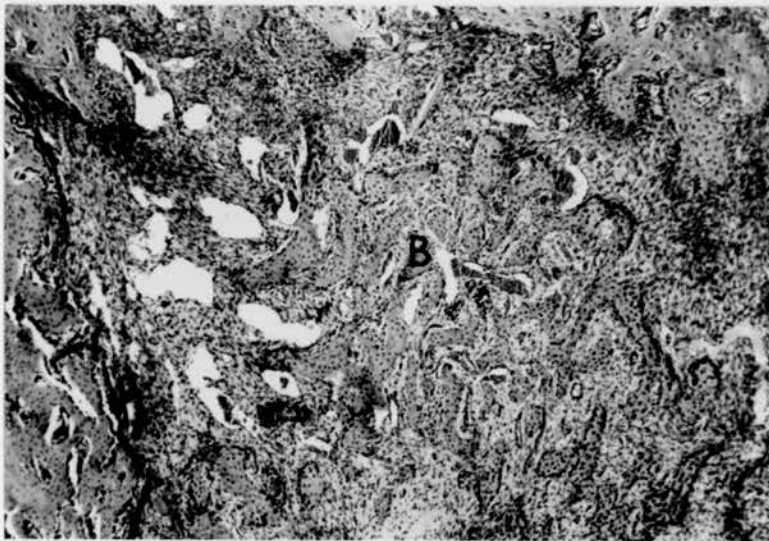
(a) Section shows fine trabeculation of bone (B) within granulation tissue which occupies the area of bone resection.

H & E x 40

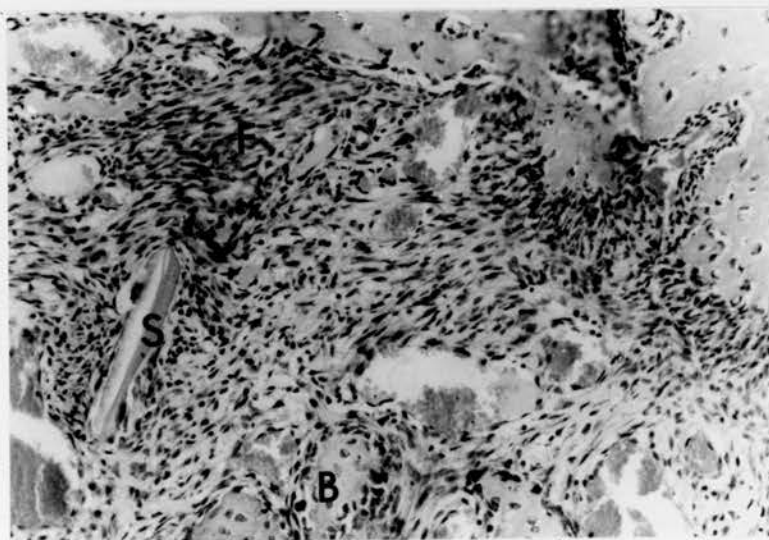
(b) Fast maturing fibrous tissue (F) within the granulation tissue. A post surgical spicule (S) of bone is identified as well as advancing trabeculae of new bone (B).

H & E x 144

Fig. 16



(a)



(b)

Fig. 17 **14 days - collagen implant**

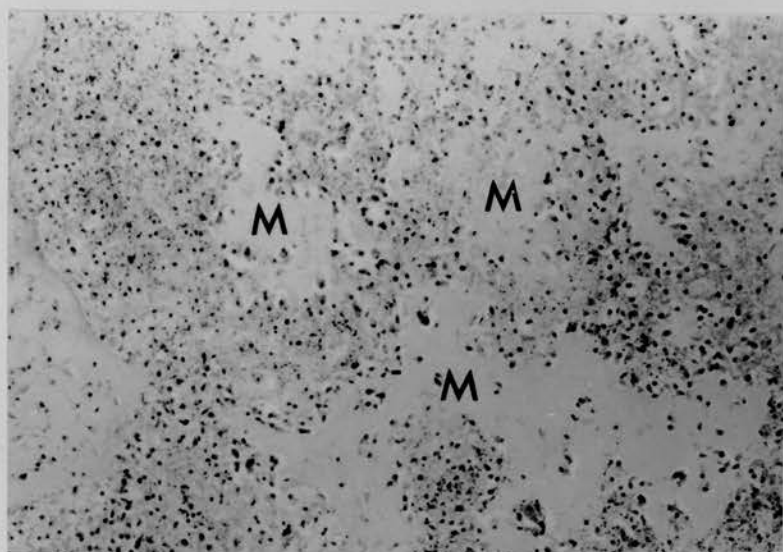
- (a) Section shows fragmented implant material (M) partially replaced by granulation tissue.

H & E x 40

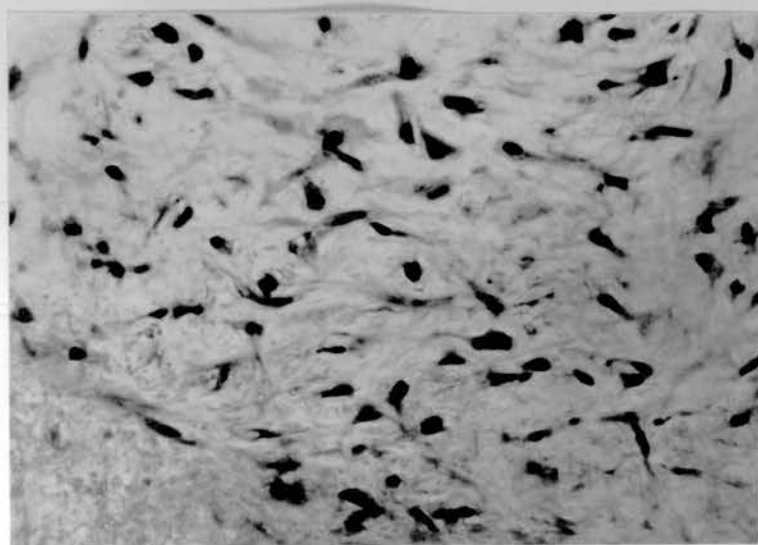
- (b) High power view shows early fibrocytes (cf. fibroblasts) swarming over and through fragmenting implant material. (Note absence of foreign body giant cell reaction.)

H & E x 360

Fig. 17



(a)



(b)

Fig. 18 **14 days - control**

Comparable control shows much more mature fibrous tissue (F) as demonstrated by cell cohesion and regular orientation.

H & E x 40

Fig. 18

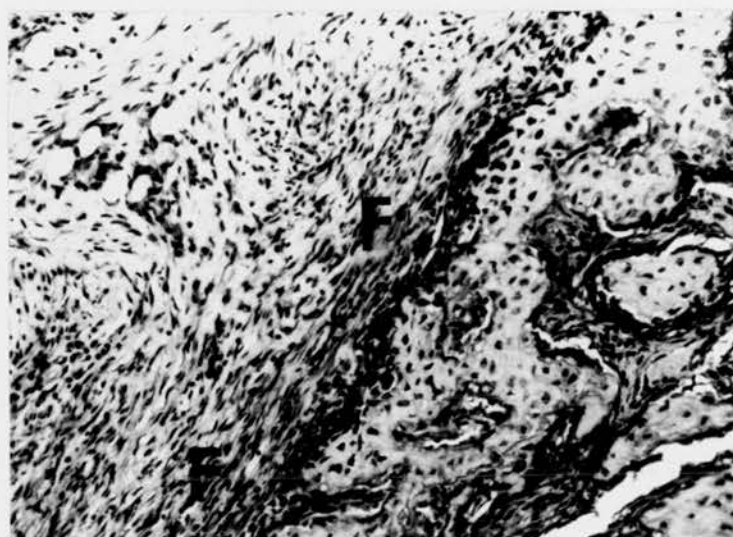


Fig. 19 **21 days - collagen implant**

Advancing bone front shows progression to lamellar bone (B) and minimal bone trabeculae but occlusion of surgical defect by retained implant material (M).

H & E x 96

Fig. 20 **21 days - control**

Nearly complete occlusion of surgical defect by granulation tissue (G) and plentiful trabeculae of bone (B).

H & E x 144

Fig. 19

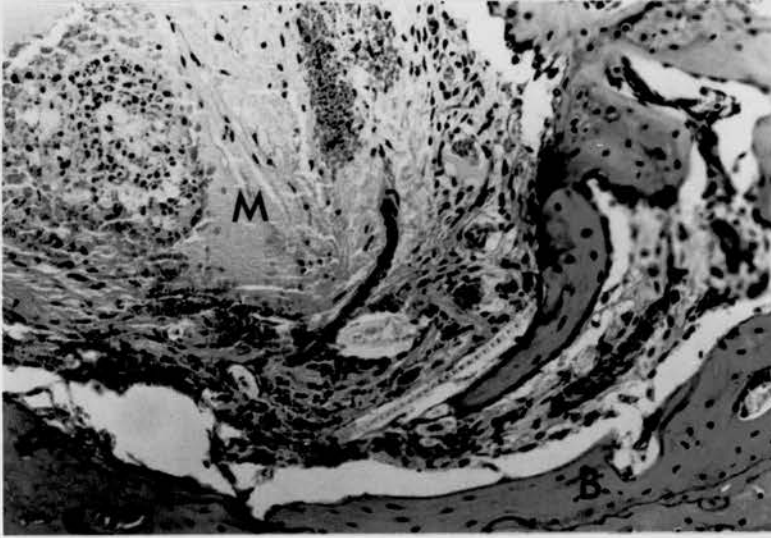
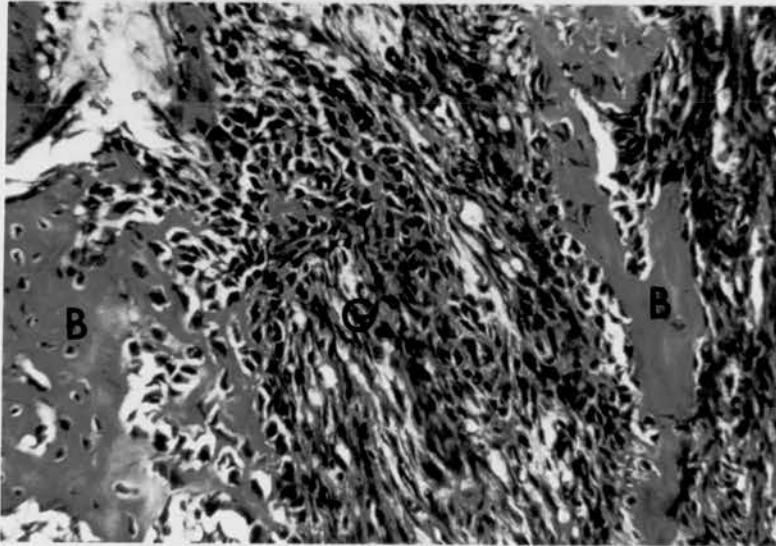


Fig. 20



The following six colour illustrations show the colour banding associated with tetracycline (yellow band T, day of operation), haematoporphyrine (orange band H, 7 days) and tetracycline (yellow band B, 14 days).

Fig. 21 21 days - control

(a) Regular advancing trabeculae of new bone.

x 144

(b) High power view of advancing front of osteoblasts.

x 320

(c) Lacunae of maturing bone showing incremental pattern of ossification.

x 560

Fig. 21

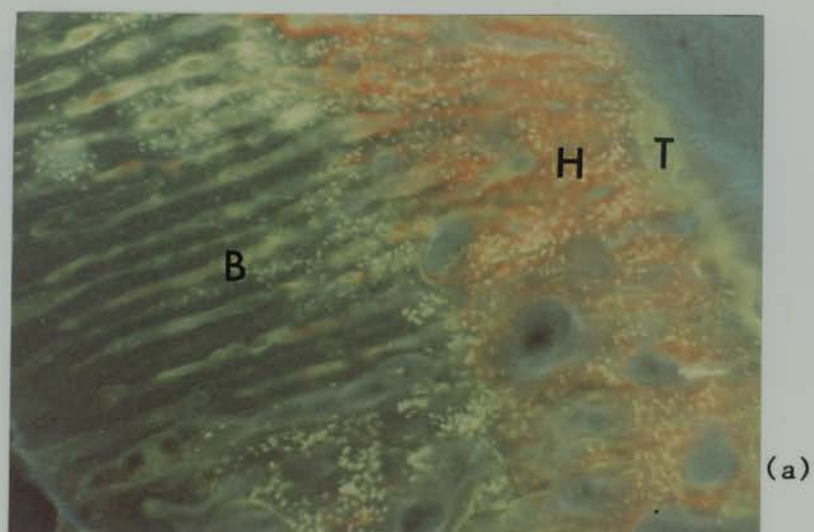


Fig. 22 21 days - collagen implant

- (a) Compared to Fig. 9a the advancing bone is less well orientated and lacks the fine trabeculation. Amorphous implant material (M) is identified.

x 144

- (b) Slower rate of replacement of implant material by bone is evidenced by the lack of fine trabeculation. Area A Fig. 22a.

x 300

- (c) Lacunae again show incremental pattern of ossification demonstrating the underlying process has not been fundamentally altered only retarded.

x 560

Fig. 22

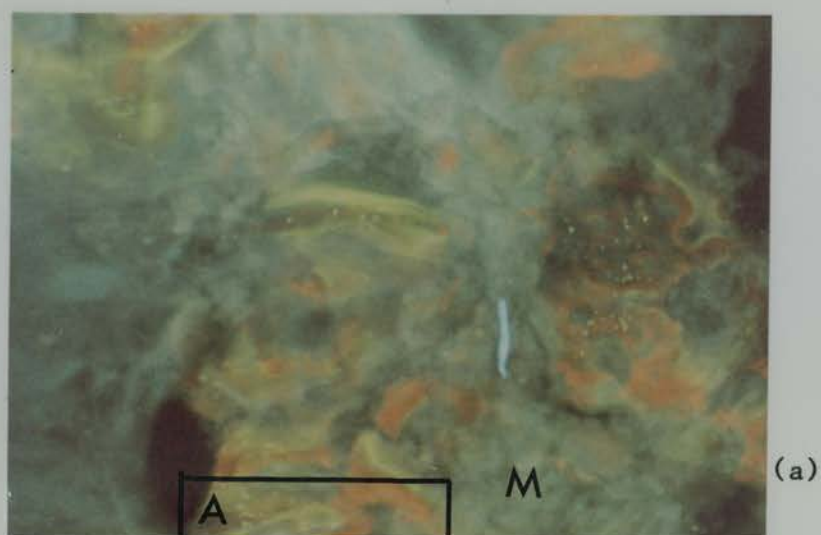


Fig. 23 **28 days - collagen implant**
Surgical defect still present.

H & E x 40

Fig. 24 **28 days - control**
Bone trabeculae (B) now shown to extend throughout the
surgical defect.

H & E x 40

Fig. 23



Fig. 24

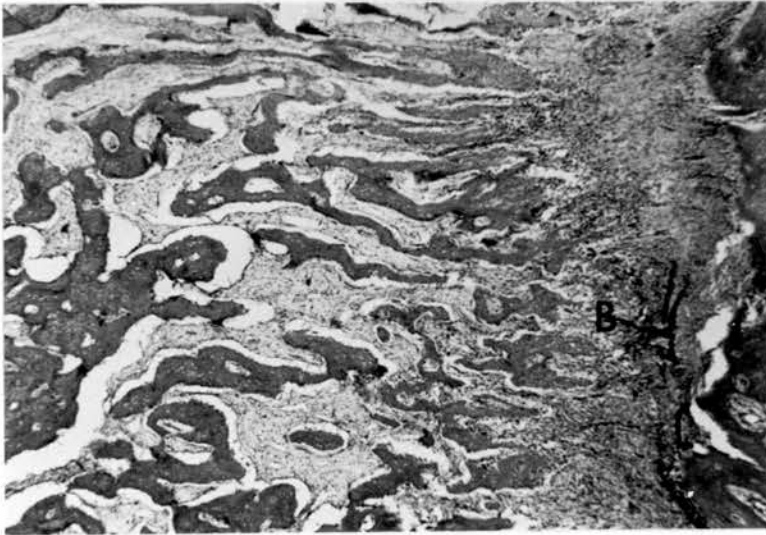


Fig. 25 **28 days - control**

(a & b) Tetracycline only. High and low power views show continuation of more regular organised ossification occluding the surgical defect.

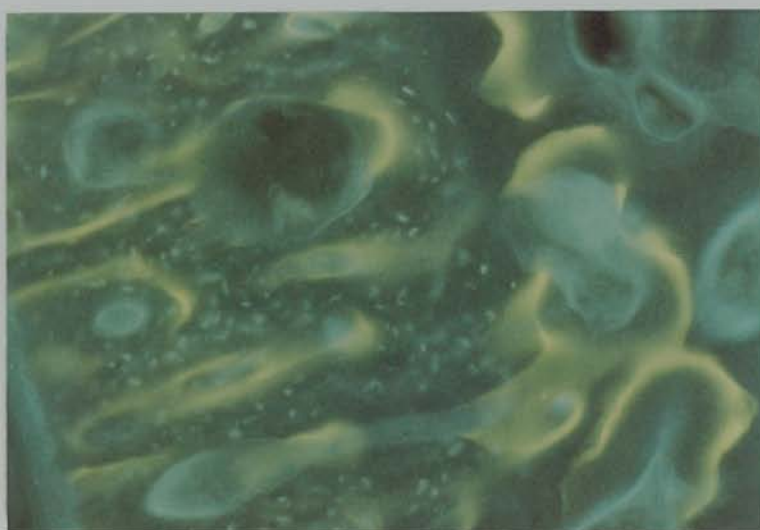
a x 144

b x 560

Fig. 25



(a)



(b)

Fig. 26 28 days - collagen implant

At 28 days the presence of the implant material (M) still retards healing and the pattern of ossification retains a more disorganised pattern.

x 144

Fig. 26

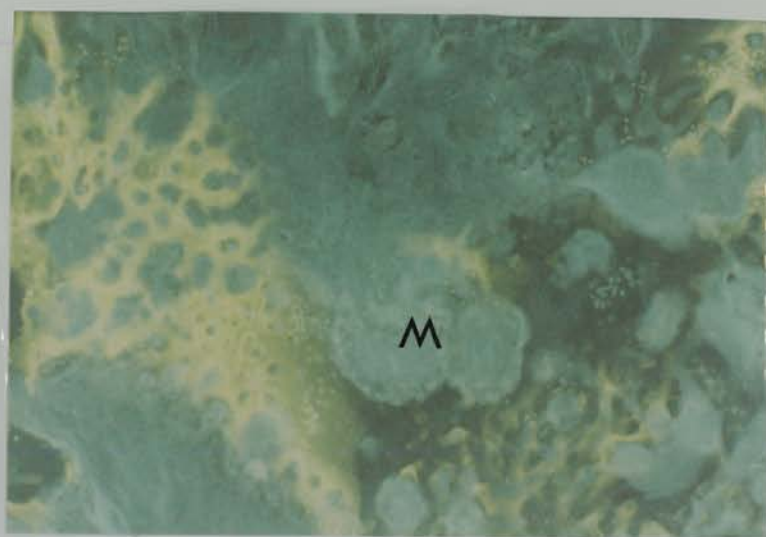


Fig. 27 35 days - collagen implant

Low and higher power views (a) and (b) show woven bone (B) still forming along with fibrous granulation tissue (G). N.B. Implant material is now no longer evident.

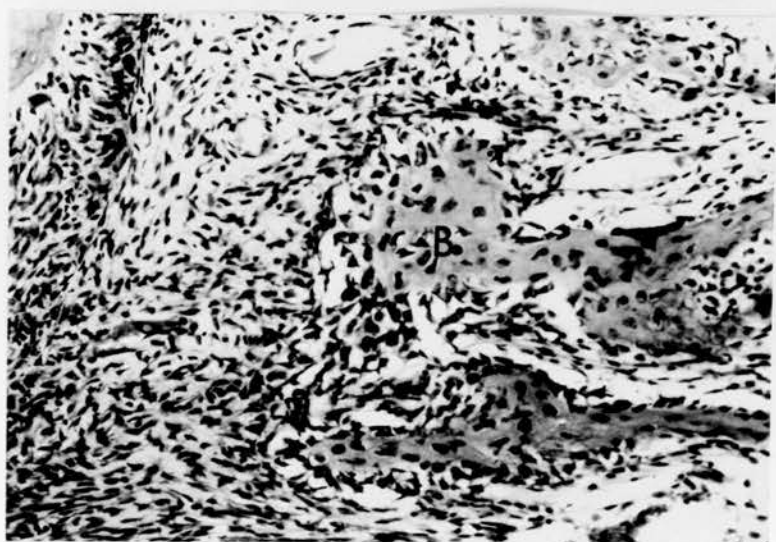
(a) H & E x 40

(b) H & E x 144

Fig. 27



(a)



(b)

Fig. 28 35 days - control
Bony replacement of surgical defect.
H & E x 96

Fig. 28

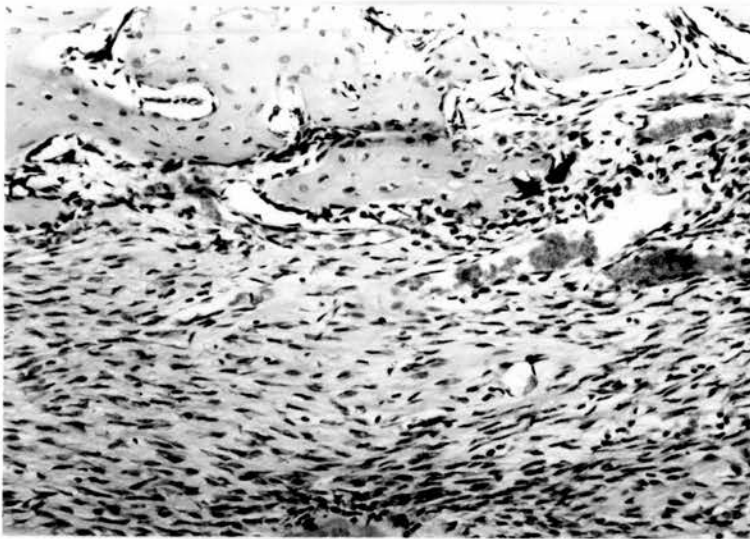


Fig. 29 35 days - collagen implant
(a) and (b) confirm the more disorganised and localised
(b) areas of ossification compared with the more
advanced and regular patterns seen on the control side.

 (a) x 40
 (b) x 144

Fig. 29



(a)



(b)

Fig. 30 **35 days - control**

In centre of original defect there is now an organised and more regular pattern of ossification with total replacement of the surgical defect.

x 144

Fig. 30

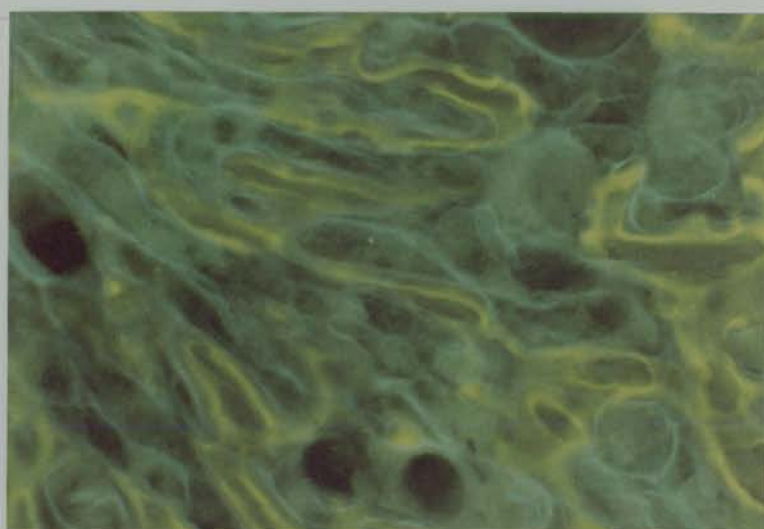


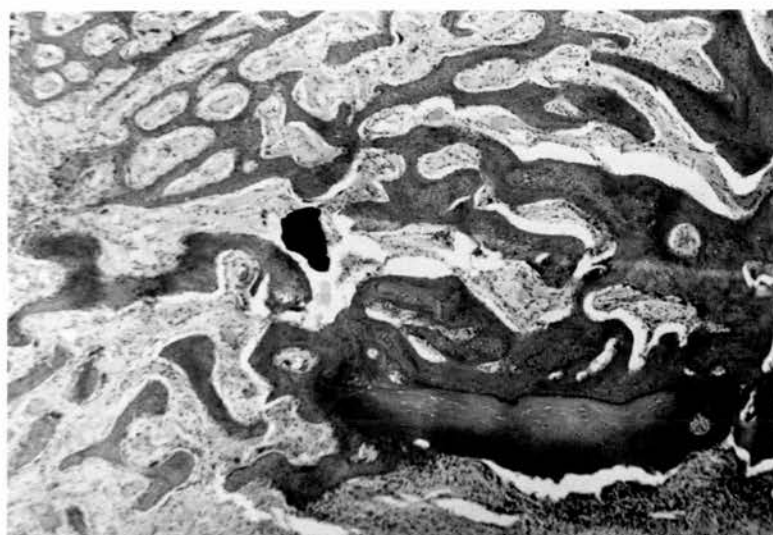
Fig. 31 84 days - collagen implant

At 84 days a comparable bony trabecular pattern to that seen in Fig. 16 (35 days) on the control side is now evident.

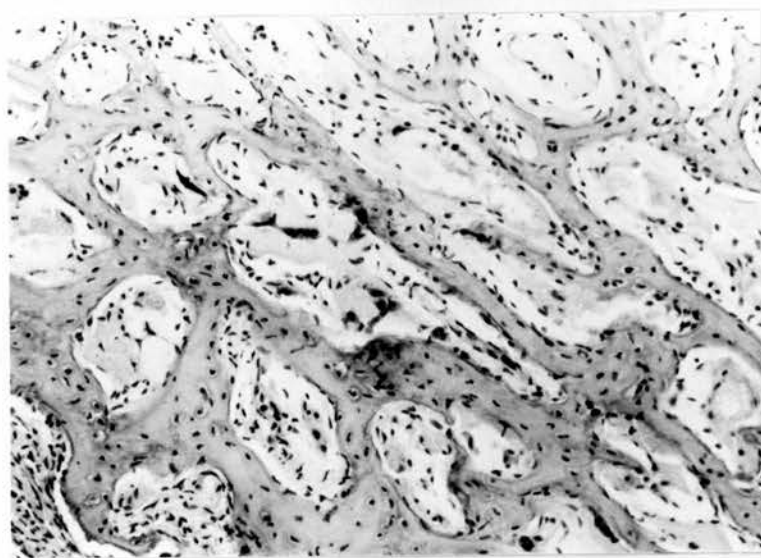
(a) H & E x 60

(b) H & E x 144

Fig. 31



(a)



(b)

d) **The quantitation of immunoglobulins specific to collagen implant matrix**

In addition to the histological examination of cell types, the immune response was demonstrated directly by monitoring antibody levels by an ELISA.

Serum taken from animals A27 and A32 was used in the study. Samples were taken at the time of operation (week 0) and at weekly intervals up to 12 weeks. Aliquots of each serum sample were mixed and used as a combined reference serum.

The positive change from colourless to yellow indicated the presence of antibody specific to the implanted collagen. Dilutions of serum from 1 to 1/2187 were used. Control wells and those from week 0 showed no colour change (Fig. 32).

Absorbance values were measured from 30 minutes to 120 minutes at intervals of 15 minutes. Optimal readings were recorded at 120 minutes. A curve for the reference sera plotting optical density against serum concentration was compiled (Fig. 33a). Using this curve at concentrations of 1/27 and 1/81 the optical density was given an arbitrary value of 100 ELISA units. The weekly serum samples were given values for their optical densities in ELISA Units at the same concentrations of 1/27 and 1/81 with reference to the standard value of 100 units.

An actual set of values obtained is shown in Fig. 33b. Values for the four occasions the experiment was repeated were found to be very similar.

Although serum samples from two animals were used and the antibody response theoretically would be unlikely to be identical, it is of interest that the two halves of the response shown in

Fig. 33b are complementary in forming one confluent curve.

The antibody response shown by the animal A32 appears to be immediate with a high level of detectable antibody present by the end of week one. The levels then began to fall sharply after week 1 until sacrifice at week 6. Animal A27 (weeks 7 to 12) showed an initial response at week 7, less than the previous level for animal A32. The level of detectable antibody then fell sharply to week 9 and then gradually tailed off in the last three weeks.

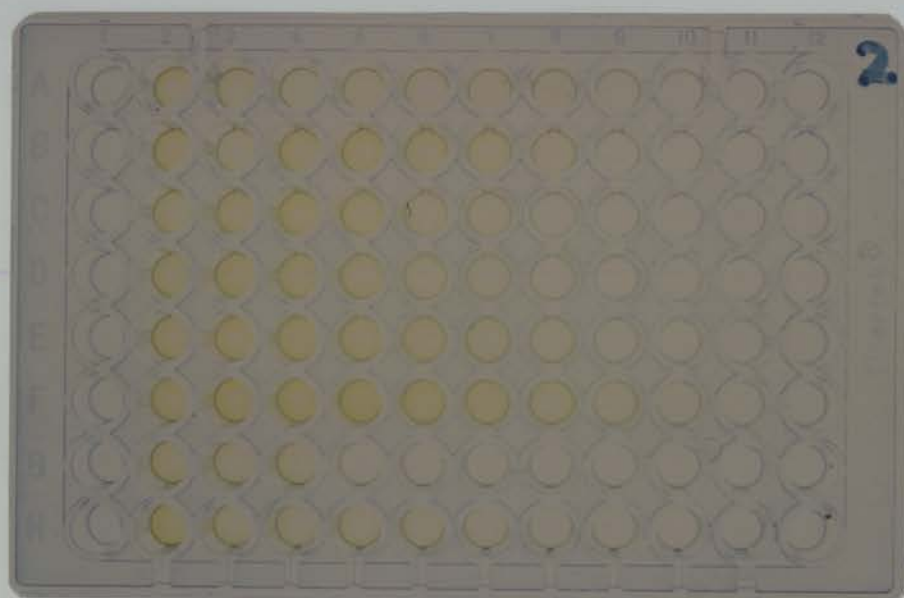


Fig. 32 Flat bottomed polystyrene microtitration plate showing colour change from colourless to yellow (positive result) from which absorbance is measured by Titertek Multiskan.

Fig. 33 Antibody Response to Collagen Implant**(a) Reference sera**

A set of values of optical densities (O.D.) recorded for serial dilutions of the combined reference sera using a semi-log scale, is shown. Readings for O.D. at dilutions of 1/27 and 1/81 are marked.

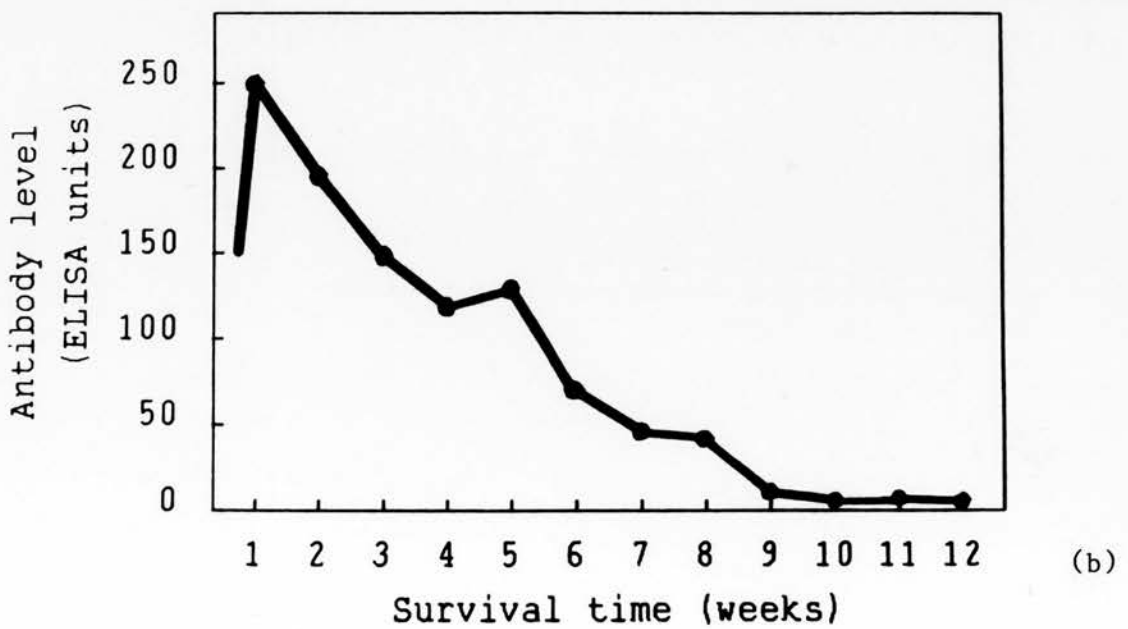
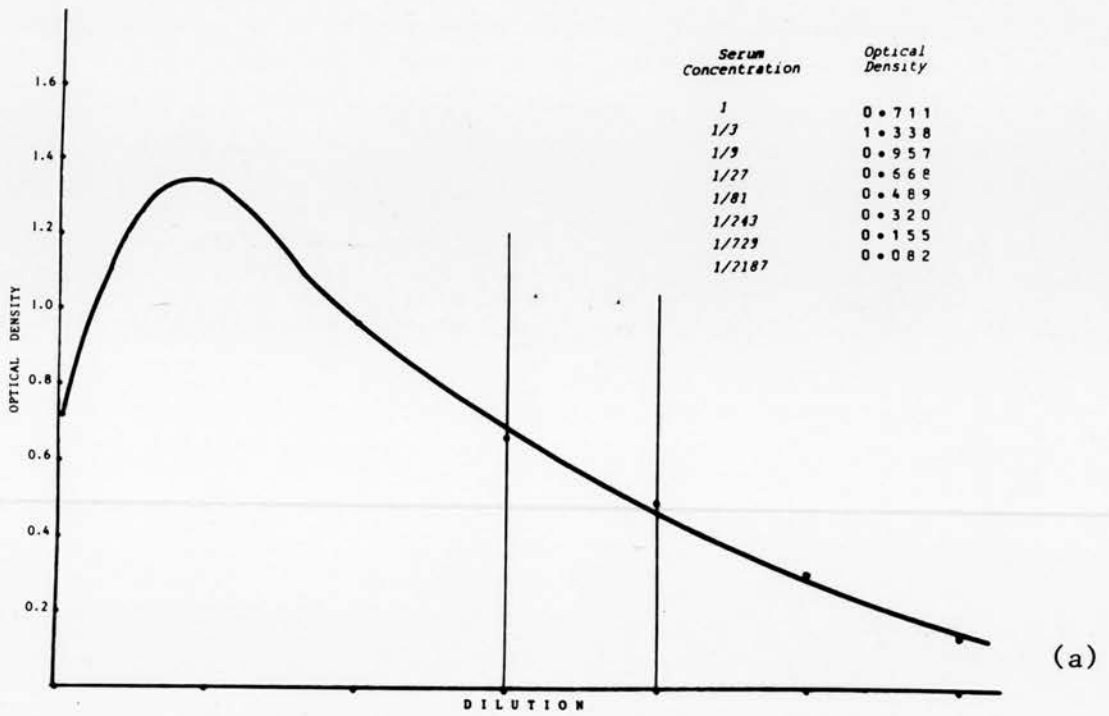
The O.D. for these dilutions is given an arbitrary value of 100 ELISA units.

(b) Antibody response

Each sample of rabbit serum, removed at weekly intervals, is serially diluted. O.D. readings for each sample at dilutions of 1/27 and 1/81 are recorded. With reference to the O.D. of the combined reference sera above, given a value of 100 units at these dilutions a value is recorded for each serum sample.

These values are plotted against time (in weeks) to demonstrate the antibody response.

Fig. 33



e) Radiology of rabbit mandibles

Radiological changes at operation sites in the rabbit were used to confirm the findings of the fluorescent bone labelling study and to show how early these effects could be seen radiographically. They were further used to confirm the extent that mandibular architecture could be restored.

Radiographs taken of mandibles up to 10 days after surgery showed no detectable changes in the radiolucent areas. The cut margins of the defects were clearly defined and the radiological density seemed unchanged (Figs. 34 & 35). At 10 days (animal A12) the margins of the lesions on the control side appeared to be less distinct when compared with the implanted side (Fig. 36).

At 14 days (animal A2) (Fig. 37) the implanted area still demonstrated clear cut margins with no change in the density of the lucent zone, while the control area had lost the clear margins and the density of the area showed an increase in opacity.

At three weeks (animal A5) (Fig. 38) the control area showed more marked opacification and the lower border of the right mandible was becoming reconstituted. By comparison, the implanted area showed that the clear cut margins were becoming rounded off, the defect was still very obvious but an increase in radiodensity was noted. The lower border of the mandible was not reconstituted.

By four weeks (animal A19) the site of operation was less obvious on the implanted side (Figs. 39) with a marked increase in radio-opacity and the lower border of the mandible was beginning to be reformed. The control side again showed a more advanced state of healing with the lower border then in continuity.

Figs. 40 indicated the picture at five weeks (animal A15). Bony regeneration at the apices of the roots sectioned at operation was seen at both control and implant sites. In this animal reconstitution of the lower border is more advanced on the implanted side compared with the control.

By six weeks (animal A32) (Figs. 41) there was no further change on the implanted side from Figs. 39 at four weeks with the lower border as yet not restored. The degree of bony replacement was still far behind that seen on the control side.

By 12 weeks (animal A27) post-operatively both sides of the operated mandibles had filled with bone and the lower borders were reconstituted. There was, however, "notching" of the replaced lower border which seemed more pronounced on the side that was implanted with the collagen matrix.

Fig. 34 5 days: No radiological changes, cut margins still clear, defects still radiolucent.

Fig. 35 7 days: No radiological changes.

Fig. 36 10 days: Cut margins now becoming less distinct, especially the control (L) side, with reduction in radiolucent area.

R (implant)

L (control)



34



35



36

Fig. 37 2 weeks: Control (L) side shows opacification of the defect. The implanted (R) side appears more radiolucent and the margins are still quite distinct.

Fig. 38 3 weeks: On the control (L) side there appears to be calcification in the defect with the lower border of the mandible almost reconstituted. The (R) side shows increased opacification, the cut margins being rounded off but the defect is still obvious radiographically.

Fig. 39 4 weeks: Control (L) side shows new bone deposited around the apices of the cut teeth. The lower border of the mandible is in continuity. The implanted side (R) shows increased opacification with bone regeneration but the defect is still visible and the lower border of the mandible not yet restored.

R (implant)

L (control)



37



38



39

Fig. 40 5 weeks: Control (L) side shows bony regeneration at the apices of the cut teeth but in this animal the lower border is not yet reconstituted. On the implanted (R) side, there is also bony regeneration in the defect and the lower border of the mandible is being reconstituted.

Fig. 41 6 weeks: The control (L) side shows recontour of the mandibular defect with good bony deposition. Some "notching" in the lower border is noted. The implanted (R) side shows bone deposited around the cut apices but the defect is still obvious and the lower border is not reconstituted.

Fig. 42 12 weeks: Both sides show the defects have now been filled with bone and the lower borders reconstituted. The bone on the control side (L) would appear to be denser than the (R). There is "notching" in the lower border on both sides but this is more pronounced on the implanted (R) side.

R (implant)



L (control)



40



41



42

3. Human Implantation Study

The safety and efficiency of the collagen implant for human use was assessed clinically and radiographically after implanting the material into cavities, created by disease, in the maxilla and mandible.

Pathological cavities may breach both cortical plates on only the buccal or lingual cortex; the original radiological density of the area is different in the two situations. When both cortices are fenestrated the area is totally radiolucent but where only one plate is intact, there remains a radiopacity depending on the thickness of bone remaining. It was assumed that the collagen matrix would not change the radiopacity of the bony cavity. To verify this, cavities were cut in a pig mandible, two fenestrating both inner and outer cortical plates and two cut only into half the mandibular width. Collagen was placed in one of the full thickness cavities and one of the partial thickness and the others used as controls. Radiographs of the mandible (Fig. 43) showed the comparable radiological densities of these cavities. There is an obvious radiological difference between a cavity which in depth is the whole thickness of the bone and that of half the mandibular width. Little radiological difference is detected between such cavities containing the collagen implant and those without. A change in radiological density of the operated area is therefore of significance as it would indicate that the area is being replaced by a more radio-opaque, calcified matrix.

a) Radiological variation

When radiographs of the healing bony cavities were compared, some variations within the implanted and control groups were found. There were also similarities between the two groups. The following examples are used to show the variations seen and they are then used to demonstrate the similarities and differences between the two groups.

A typical cystic area, as indicated in Fig. 44a, is seen to be well circumscribed with a well defined margin. The cyst has been enucleated, the cavity filled with haematoma and allowed to heal. Six months after enucleation, radiating trabeculae of new bone are seen from the periphery of the original area towards the central zone (Fig. 44b) which in comparison now appears more radiolucent. The well circumscribed original margin by this time has been lost. At one year post-surgery, the original area is almost completely replaced by bone of repair (Fig. 44c). In comparison a similar state of healing can also be demonstrated (Figs. 45a,b,c,d) when such an area has been implanted with the collagen matrix. At three months, there is a peripheral radiopaque zone surrounding a more radiolucent central area (Fig. 45b). By six months, this peripheral zone shows the radiating trabeculae of new bone formation while the central area is still radiolucent (Fig. 45c). At one year (Fig. 45d), the original area has filled with bone of repair.

Figs. 46, 47 and 48 relate to the control group and indicate some of the variants seen radiographically in the healing pattern. Areas may fill with bone of repair (Fig. 46) but leave a small radiolucency adjacent to the seal placed in the apex of an adjacent root, possibly a fibrous barrier. This is also demonstrated with a

larger initial area (Fig. 47) when such a radiolucent residue may still be detectable two years post-surgery. In larger areas, when the original bony defect, in part, is through the full thickness of the bone, breaching both cortical plates, then, although reparative bone can be seen in the peripheral area the central area (with full thickness defect) still remains as a radiolucent zone which may be permanent (Fig. 48). It is assumed that as such areas are symptom-free healing has taken place by fibrous tissue alone. These areas do not calcify nor are they replaced ultimately by bone.

Similar variants have been noted in the implanted group. In Fig. 49 bony repair has taken place at the periphery of the original lesion but the central zone has remained as a well circumscribed radiolucency. This situation thus compares directly with Figs. 48. A comparison of Figs. 50 and 46 can be made when, although healing of the bony defect has taken place, a well circumscribed lucent area still remains around the apices of roots involved in the original area. Figs. 51 and 47 are also directly comparable demonstrating at two years post-surgery a small lucent area related to an apical seal is still present. It can therefore be inferred, with the examples used, that a similar healing process shown radiographically can take place in both control and implanted groups with similar variations.

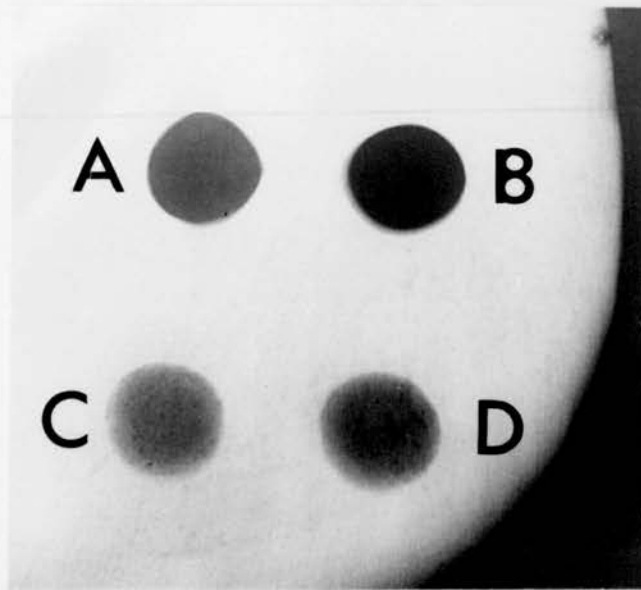


Fig. 43 Pig mandible to show density of defects with and without collagen paste.

- A Cavity full thickness of mandible + collagen.
- B Cavity full thickness of mandible.
- C Cavity half mandibular thickness + collagen.
- D Cavity half mandibular thickness.

- Fig. 44** Healing of non-implanted cystic lesion of the maxilla:
- a) Original well-circumscribed cystic lesion.
 - b) Six months post-cyst enucleation showing radiating trabeculae of new bone radiating in from periphery of original area towards central zone. Note also loss of well-circumscribed margin.
 - c) Original area now almost completely replaced by radiating bone of repair.

- Fig. 45** Healing of cystic/granulomatous lesion of the mandible with implant:
- a) Shows extent of original lesion.
 - b) Three months post-operatively demonstrating a peripheral radio-opaque area with a more lucent central area and margin of lesion still demonstrable.
 - c) Six months post-operatively shows that the peripheral zone is replaced by radiating trabeculae of bone, the original margin has become lost and a small lucent area remains centrally. Small fragments of amalgam used as an apical seal are seen.
 - d) At one year the whole area has healed with bone.

Fig. 44



(a)

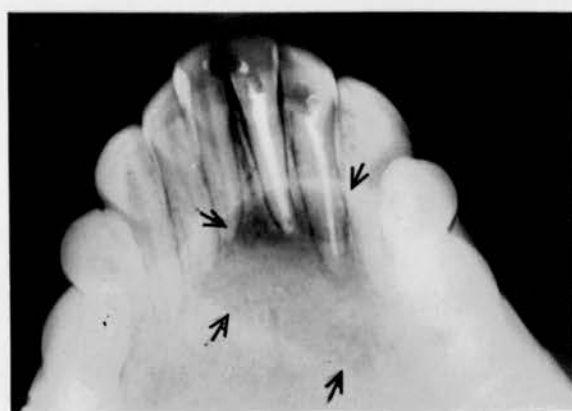


(b)



(c)

Fig. 45



(a)



(b)



(c)



(d)

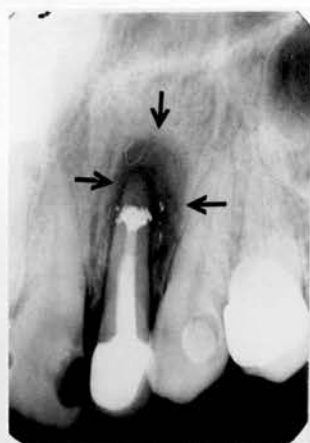
Radiographs to demonstrate classification of lesion by size and show the healing process. Magnification of the area by the radiograph is taken into account when estimating the size of the bony defect.

Fig. 46 (a) S (small) area <1 cm in diameter.
 (b & c) Gradual replacement of the area with trabeculated bone over one year. Note small radiolucent area in C adjacent to amalgam seal at root apex. Probably this area remains as a fibrous seal and does not calcify. (Control group)

Fig. 47 (a) M (medium) area 1-2 cm in diameter.
 (b & c) Again demonstrate bony repair and (c) shows again a small lucent area at root apex two years after surgery. (Control group)

Fig. 48 (a) L (large) lesion 2 cm + in diameter.
 (b) At one year shows radiating trabeculae of new bone filling more than half the original defect but a central lucent area still remains. (Control group)

Fig. 46



(a)



(b)



(c)

Fig. 47



(a)



(b)



(c)

Fig. 48



(a)



(b)

Radiographs show cases where clinically healing has taken place, but radiographically the area has not been fully replaced by bone.

Fig. 49 (a) Maxillary cyst removed from an area where supporting bone has been lost through the whole depth of the maxilla. Bony repair has taken place, (b) but a well demarcated area at apex of root shows as a radiolucency. This area where there has been loss of the whole depth of the maxillary bone is healed probably by fibrous tissue alone. (Collagen implant group)

Fig. 50 (a) Shows apical cystic granulomatous lesion. (b) Shows healed lesion with residual radiolucent area around apices of adjacent teeth after one year. (Collagen implant group)

Fig. 51 (a) Shows early peripheral radio-opaque zone progressing from original margin (arrowed) three months post-surgery. (b) At two years the original defect has become replaced by bone but a small lucent area related to the tooth apex is noted. (Collagen implant group)

Arrows indicate extent of original lesions.

Fig. 49



(a)



(b)

Fig. 50



(a)



(b)

Fig. 51



(a)



(b)

b) Patient progress

The progress of patients allocated to each group is shown in Tables 6.2 to 6.7 and individual patient progress shown in Appendix 1. The healing of bony cavities in the jaws of man with a collagen implant and in a control group was shown radiographically with comparable films taken for each patient at intervals of three, six and 12 months.

Tables 6.2 and 6.5 show that groups were comparable. The allocation for the implant group was 19 males and 31 females, compared to the control group of 22 males and 28 females.

In the implant group, 13 areas were present in the maxilla in the males and six in the mandible compared with 17 and five in the control. Twenty four areas were present in the maxilla in the female implant group and seven in the mandible, compared with 21 and seven in the control group.

Tables 6.2 to 6.4, show the progress of the collagen implanted group. At three months, seven of the group did not return and from the remaining 43, only 14 showed increased opacification of the original area. By six months, however, of the 41 patients who returned for review, 38 had evidence of some new bone present within the original healing area. At one year only 37 patients returned; 22 showed complete healing of the area with bone of repair being demonstrated. In another 14 cases, the areas were partially replaced by bone with central lucent areas still remaining (Figs. 48 & 49). One patient showed a radiolucent area which had not changed, and although the patient was clinically symptomless, radiating trabeculae of new bone were not demonstrable; it must be assumed that healing was by fibrosis only.

The results for patients in the control group are shown in Tables 6.5 to 6.7. At three months, nine patients failed to return and 32 of the remaining 41 patients showed radiographically by an increasing opacification that healing was progressing. In many instances there was loss of the original clean cut margins. At six months a figure similar to that at three months of 32 from 39 patients returning demonstrated evidence of healing progressing by the deposition of new bone. At one year only 35 patients returned; 27 showed the original lesion to be fully healed and replaced by bone. A further five showed bony repair in part of the original lesion and three showed no evidence of repair by bone.

It is noted in Appendix 1 that two patients KC and KV in the control group required the original defects to be re-explored due to persistent residual infection.

For descriptive purposes and for comparison, the sites of operation used in this study have been divided into three groups with an allowance made for magnification of the films. Areas <1 cm in diameter (S group) are shown in Fig. 46; defects 1-2 cm in diameter (M group) are shown in Fig. 47, while lesions >2 cm in (L group) are demonstrated by Fig. 48.

Table 6.8 shows the state of healing seen in the control and implant groups at three months related to size of the original bone cavity. Fifteen from 20 small areas showed bony deposition in the S control group, whilst only six from 24 were seen in the implant group. The M sized areas in the control groups also showed a high rate of bone deposition, 15 from 17, whilst the implant group only showed six from 15. The large areas in both the implant and control groups showed similar figures, two from four.

By six months, Table 6.9, most of the S areas in the implant group showed evidence of bony formation, 18 from 20, and in the control group, 14 from 19. Similar figures were found in both the M and L areas for both groups, the majority of lesions showing bony deposition.

By one year similar numbers in all three groups, S, M and L showed fully healed wounds in both control and implanted groups. A difference between control and implant groups was shown in the number of areas with only partial repair by bone. In the controls, only five patients showed partial repair, one in the S group, three in the M group and one in the L group. By comparison, seven in the S group, four in the M group and three in the L group of the implanted patients only showed partial repair.

The figures in Tables 6.8, 6.9 and 6.10 comparing healing of S, M and L areas within their own groups, were found to have no statistical significance. However, if the control and implant groups are compared at three, six and 12 months, the results show a difference.

Shown in Table 6.11 at three months the control group showed bone deposition in 32 of 41 patients compared with 14 of 43 patients in the implant group. These figures are shown to be statistically significant ($P = 0.0001$) which confirms the earlier results that there seems to be a significant delay in the collagen implanted wounds during the early stage of healing. By six months, similar numbers in both groups show evidence of bone deposition and at one year the difference between the control and implant areas is again not statistically significant.

TABLE 6.2

COLLAGEN IMPLANT GROUP

3 Months

HEALING OF BONE BY SITE AND SEX

	Site	Increase in radiological density in operated area	No change in radiolucent area	Patients not returning	Total
Male	Maxilla	1	9	3	13
	Mandible	2	2	2	6
Female	Maxilla	8	15	1	24
	Mandible	3	3	1	7
Total		14	29	7	50

TABLE 6.3

COLLAGEN IMPLANT GROUP

6 Months

HEALING OF BONE BY SITE AND SEX

	Site	Evidence of deposition of new bone	No change from 3 months	Patients not returning	Total
Male	Maxilla	11	1	1	13
	Mandible	4	-	2	6
Female	Maxilla	17	2	5	24
	Mandible	6	-	1	7
Total		38	3	9	50

TABLE 6.4

COLLAGEN IMPLANT GROUP

12 Months

HEALING OF BONE BY SITE AND SEX

Site		Area fully healed with deposition of bone	Trabeculated bone of repair in part of original area	No radiological evidence of repair with bone	Patients not returning	Total
Male	Maxilla	2	5	-	6	13
	Mandible	4	1	-	1	6
Female	Maxilla	10	8	1	5	24
	Mandible	6	-	-	1	7
Total		22	14	1	13	50

TABLE 6.5

CONTROL GROUP

3 Months

HEALING OF BONE BY SITE AND SEX

	Site	Increase in radiological density in operated area	No change in radiolucent area	Patients not returning	Total
Male	Maxilla	10	2	5	17
	Mandible	3	2	-	5
Female	Maxilla	16	4	1	21
	Mandible	3	1	3	7
Total		32	9	9	50

TABLE 6.6

CONTROL GROUP

6 Months

HEALING OF BONE BY SITE AND SEX

	Site	Evidence of deposition of new bone	No change from 3 months	Patients not returning	Total
Male	Maxilla	10	2	5	17
	Mandible	4	-	1	5
Female	Maxilla	15	3	3	21
	Mandible	3	2	2	7
Total		33	7	11	50

TABLE 6.7

CONTROL GROUP

12 Months

HEALING OF BONE BY SITE AND SEX

Site		Area fully healed with deposition of bone	Trabeculated bone of repair in part of original area	No radiological evidence of repair with bone	Patients not returning	Total
Male	Maxilla	5	3	1	8	17
	Mandible	3	-	-	2	5
Female	Maxilla	13	2	2	4	21
	Mandible	6	-	-	1	7
Total		27	5	3	15	50

TABLE 6.8

HEALING OF BONE BY SIZE OF ORIGINAL CAVITY

a) Control Group - 3 months

Size of bony cavity	No change	Bone deposition	Total
Small	5	15	20
Medium	2	15	17
Large	2	2	4
Total	9	32	41

chi square = 2.97493

P = 0.2259

(b) Implant Group - 3 months

Size of bony cavity	No change	Bone deposition	Total
Small	18	6	24
Medium	9	6	15
Large	2	2	4
Total	29	14	43

chi square = 1.5569

P = 0.4591

TABLE 6.9

HEALING OF BONE BY SIZE OF ORIGINAL CAVITY

a) Control Group - 6 months

Size of bony cavity	No change	Bone deposition	Total
Small	5	14	19
Medium	1	15	16
Large	1	3	4
Total	7	32	39

chi square = 2.52513

P = 0.2829

(b) Implant Group - 6 months

Size of bony cavity	No change	Bone deposition	Total
Small	2	18	20
Medium	1	16	17
Large	0	4	4
Total	3	38	41

chi square = 4.65141

P = 0.3250

TABLE 6.10

HEALING OF BONE BY SIZE OF ORIGINAL CAVITY

a) Control Group - 12 months

Size of bony cavity	No evidence of repair	Partial repair by bone	Area fully healed with bone
Small	2	1	11
Medium	0	3	14
Large	1	1	2
Total	3	5	27

chi square = 4.65141

P = 0.3250

(b) Implant Group - 12 months

Size of bony cavity	No evidence of repair	Partial repair by bone	Area fully healed with bone
Small	1	7	11
Medium	0	4	9
Large	0	3	2
Total	1	14	22

chi square = 2.30805

P = 0.6793

TABLE 6.11

HEALING OF BONE COMPARING CONTROL AND IMPLANT GROUP

a) 3 months

Group	No change	Bone deposition	Total
Control	9	32	41
Implant	29	14	43

chi square = 15.74391

P = 0.0001

(b) 6 months

Group	No change	Bone deposition	Total
Control	7	32	39
Implant	3	38	41

chi square = 1.20790

P = 0.2717

(c) 12 months

Group	No change	Partial repair by bone	Area fully healed with bone	Total
Control	3	5	27	35
Implant	1	14	22	37

chi square = 5.72220

P = 0.0572

CHAPTER 7

DISCUSSION

Restoration of the aesthetic and functional properties of bone is the aim of the maxillofacial surgeon when there is loss of facial architecture. Vascularised bone grafts replace the lost tissue with a similar viable identical matrix and may be satisfactory in restoring the local contour. Autogenous non-vascularised grafts also show a high rate of success, restoring contour and consistency but they are totally dependent on the local blood supply for survival. A secondary operation is always required to harvest these graft materials.

The use of homologous and heterologous prepared bone has met with a limited rate of success. The local vascular supply is of great importance, but equally the foreign matrix may stimulate an immune response to any trace of foreign protein components in the material.

Non-biological allogenic grafts which do not contain protein residues will preclude humoral immunological effects. The results with metallic prostheses and ceramics have been varied. These materials replace the mineral component of bone and some are said to be biocompatible, i.e. capable of coexistence without harm to the host in a manner similar to natural host tissues. Some are claimed to be biodegradable, i.e. able to be digested by normal host enzymes or disposed of by cellular elements and replaced by natural host tissue. Few are both biocompatible and biodegradable. Metallic prostheses provide strength and aesthetics when used to

replace segments of mandible but they may eventually become extruded. The ceramic materials which have excited much interest recently are claimed to be biocompatible, some are biodegradable but there is debate on the extent of host tissue penetration and replacement of these materials.

An ideal allogenic material has yet to be found. Collagen, which forms the major protein mass in bone matrix, in a heterologous form could theoretically offer an alternative approach by providing a biological matrix which is both biocompatible and biodegradable. If this were correct, it could be supplied in various forms, with differing properties, to temporarily replace a range of lost tissues. A biodegradable scaffold could be used to replace bone and if, as is suggested by some authors, that collagen itself calcifies (Shoshan & Finkelstein 1970) or increases the rate of wound healing (Solomons & Gregory 1966), then additional strength could be provided earlier during bony repair. As the material is incorporated it would eventually be replaced by host bone during the normal revision of local bony architecture.

Theoretically this would be possible only if the implant collagen had very specific properties. As discussed in Chapter 1, the resorption of a collagen matrix is variable depending both on the local tissue environment and its own physical properties. Soluble collagen with little cross linking is removed relatively quickly. The rate of elimination of collagen decreases with the degree of cross linking and highly cross linked collagen becomes extremely brittle and may not be degraded at all. The animal work on which many of these observations have been made, have shown species specificity. There have also been variations in behaviour

of the materials at different sites of implantation and the results differ with a change in the source of the collagen.

This thesis has considered all the factors outlined in Chapter 1 to form a working hypothesis that a combination of a soluble collagen with a lightly cross linked material could improve healing in areas where bone matrix is lost, and could maintain the local tissue contour. The soluble component should be easily eliminated to allow rapid replacement by host repair tissue; the insoluble portion would be maintained as a scaffold to support and guide the repair tissue to ensure total restoration of the bony architecture. If the collagen itself did calcify, then an early increase in strength at the site of repair could be expected. Heterologous collagen is a foreign protein but as all mammalian collagens exhibit a similar structure differing mainly in the constitution of the non-helical portions, they are regarded by many authors as only "weak" antigens and should therefore be safe materials to use for implantation. This study has attempted to show the effects and subsequent replacement by host tissue of such a specially formulated bovine collagen. Its desired properties should be that it is shown to be biocompatible; it should be totally resorbed, replaced or incorporated into the host with no detrimental effects. It should be non-antigenic and improve or accelerate healing. Its physical properties should be such that it is easy to manipulate; is stable in tissue fluids and shows no volume change.

To assess these theoretic possibilities, the thesis has shown the effects of the material at cellular level on human oral fibroblasts and followed the effect on healing in a prepared bony cavity in the rabbit mandible. These effects have been compared

with a control area using a longitudinal histological study, paralleled with bone labelling and radiology. The immunological response has been viewed histologically and the B lymphocyte activity shown by measuring, with an ELISA, the antibody levels raised specifically to the implanted collagen. The preliminary results from these investigations together with the results of earlier work by the author when the material had been used as a bone dressing in dry sockets (Mitchell 1986) had shown no untoward effects, and the material was therefore considered safe and suitable for use in a human implantation study. In this study the material was placed in bony cavities in the jaws after the local diseased tissue had been removed. It was considered that small areas should be treated initially so that should any adverse local effects due to the material be noted, the situation could be resolved without detriment to the patient. This study demonstrated the behaviour of the material as a bone implant.

The results of this research will now be discussed in detail.

The behaviour of the material when implanted into cell cultures of human oral fibroblasts confirmed its compatibility. More specifically, all cells in the study showed a normal shape and complement of intercellular organelles. The indented nucleus seen in Fig. 8 is not an uncommon finding on tissue culture and taken with an overall picture of healthy cells does not appear to have any significance. The fibroblasts were invariably seen to be in an actively dividing phase which was confirmed by the comparative lack of rough endoplasmic reticulum which would be abundant in cells actively producing tropocollagen. There was little doubt therefore that the collagen fibrils seen in the figures were the implant

material alone. An intimate relationship must have developed and been maintained between the implant material and the fibroblast colony as the material seemed adherent to the cell layer and did not separate when the material was processed for microscopy. This symbiotic relationship would suggest that the material should have no adverse effect at a cellular level during wound healing. The absence of cytopathic effect further confirmed the material suitable for implantation. This method was simple, easy to perform and sensitive (Autian 1970).

The prepared cavity cut in the lower border of the rabbit mandible was used to compare healing at a site implanted with the collagen matrix to one which was initially filled only with blood clot. In the control area the blood coagulum would be replaced by ingrowth of osteogenic granulation tissue and lysis of the clot would immediately precede this advancing healing tissue. In the area implanted with collagen, it was considered that the provision of a biological scaffold could increase the rate of wound healing. Such an increase had been shown by Shoshan & Finkelstein (1970) when ^3H proline labelled collagen was implanted into muscle wounds in the guinea pig back. It was demonstrated in the guinea pig that the collagen matrix reduced the early haemorrhage and oedema which had been seen on the control side. At six days post-implantation, mature fibrocytes were seen to be producing newly deposited non-labelled collagen fibrils and the control area was retarded by comparison. By day 11 in their study, healing of the implanted area with newly formed collagen was well in advance of the control wound and by three weeks both wounds had healed, the collagen implant being almost completely replaced by new host collagen. The

control wounds, although comparably well healed, still showed microscopically areas of degenerated muscle and lymphocyte infiltration. Their conclusion was that a collagen implant had produced a beneficial effect on healing of a muscular wound in the guinea pig back. Although the guinea pig muscle implant study was not directly comparable to the use of collagen in a bony cavity, the findings of this thesis related to the early period of healing, refutes their results.

The animal model in this study showed a fibroblast rich granulation tissue, at a period of five days after surgery, surrounding areas containing the collagen matrix and trabeculae of new bone were beginning to be laid down (Figs. 13a,b,c). A very similar finding was seen on the control side except that remnants of blood clot substituted for collagen (Figs. 14a,b,c). At 10 days, the control area exhibited a mature osteogenic granulation tissue with advancing bony trabeculation (Figs. 16a,b) which seemed to be in advance of the implanted area, contrary to the findings of the guinea pig study.

At 14 days implant material was still evident, but it was fragmenting and fibrocytes were seen swarming into and around it (Figs. 17a,b). By comparison now the control area showed a regular orientation of fibrous tissue and a more mature advancing bony front (Fig. 18). It was more definite at this stage that healing of the control area was in advance of the implanted site. It was hoped that this would be further verified by the microvascular study but this was unfortunately abandoned as stated in Chapter 6 and will be discussed later.

By comparison, the results of the guinea pig study (Shoshan & Finklestein 1970) and this thesis have shown that the site of implantation must be important and muscle may have been a more suitable vascular bed for implant material. The results may have demonstrated species specificity and have shown how unreliable conclusions from animal work may be when applied to another animal or to the human situation. If we now consider the reports of Devore (1977), Fabinger et al (1980) and Joos et al (1980) who all claimed that wound healing was accelerated and improved when collagen was implanted into bone, we find that they contradict the findings of this thesis.

The latter two studies were probably not strictly comparable with this investigation. They were dealing with periodontal pockets and using the material as a bone dressing they showed that the collagen left in situ in these pockets directly calcified initially. Calcification was then followed by early penetration of fibroblasts and osteoblasts with subsequent formation of bone. The control areas also showed bony regeneration but in a haphazard fashion and to a lesser extent. Devore's study (1977) is more directly comparable with this thesis, in that it deals with similar collagen implants, the rabbit model and a bony cavity. The results of Devore's work showed that by two weeks there was little change in the grafted or control regions, progress being similar in both areas. However, by eight weeks the defect on the control sides had begun to fill but with marked notching of the inferior border of the mandible, while healing at the implanted sites was much more advanced, with little or no notching. At three months the healing at the implanted sites was still more advanced with almost complete

replacement by bone and only a minor degree of notching on the lower border when compared to a more significant notching at the control areas. A similar picture was also noted at one year. The study concluded that the cross linked collagen implant initiated or aided the total replacement of the area by host bone and healing was accelerated.

This thesis does not support the findings in Devore's study. There are features in the studies which appear similar. In this thesis the collagen matrix could be seen histologically at 28 days and was finally eliminated by 35 days. This rate of implant elimination was comparable with the material used in Devore's study at 42 days. Thus, if the time of elimination of the material is proportional to cross linking, then the material used in this research would appear similar to that used by Devore. To further complicate the situation, Colago et al (1965) showed no beneficial effect from a collagen implant, that was eliminated by 14 days, on bone healing in the rat.

It is not apparent why healing at the implanted area should be delayed in comparison with the control in this thesis, but experimental evidence by Silver (1980) suggests this should be expected. It was demonstrated that the micro-environment during wound healing shows a fall in oxygen (O_2) tension and a rise in carbon dioxide (CO_2) with increases of H^+ and K^+ ion concentrations. The oxygen tension is known to be critical to wound healing, hypoxia being detrimental, as normal cellular function is dependent on more oxygen supporting pathways in preference to anaerobic conditions (Silver 1980). Indeed it has also been demonstrated that wound healing is directly related

to oxygen tension and can be improved by an increase in oxygen tension (Niinikowski 1980). In wound cavities initially the O_2 tension is low and the CO_2 tension high but with the ingrowth of granulation tissue and formation of endothelial bands, the pCO_2 decreases and the pO_2 is seen to gradually increase. The presence of foreign bodies in wounds interferes with the ingrowth of granulation tissue thereby influencing the oxygen tension and interfering with the supply of nutrients to the wound. The importance of nutrients has been demonstrated by placing in the centre of an implanted cellulose sponge a highly nutritional medium of glucose, amino acids, electrolytes and vitamins which produce a significant increase in the local deposition of collagen. It can thus be argued that the provision of a collagen matrix in this research had produced in the local wound environment an oxygen and nutritional lack which retarded the healing process. This view is perhaps supported by Cochran & Hiat (1975) who showed that bony defects in the pelvis and femur filled with collagen demonstrated comparable healing with non-implanted controls, showing no evidence of enhanced bone healing. Hunt & Benoit (1976) may further support the argument as they concluded that collagen placed in the feline tooth socket resulted in delayed bony repair.

If this hypothesis is acceptable it is not clear how Devore's study was so positive in its inferences on the positive effect of a collagen implant matrix to wound healing.

The impression of healing retardation in this study was further examined by considering the progress of the comparable bony lesions in the rabbit from the third week to the twelfth week.

At a period of three weeks after surgery, histologically the control area showed almost complete occlusion of the surgical defect with granulation tissue and plentiful bone trabeculation (Fig. 20). By comparison, the implanted site (Fig. 19) demonstrated that although healing was taking place, the presence of implant material prevented the advancing granulation tissue from completely filling the defect. Healing was therefore retarded. This situation was further exemplified by comparing Figs. 21a,b,c (control) with Figs. 22a,b,c (collagen implant); both are undecalcified preparations showing bone staining. It was evident from Figs. 21c and 22c that a similar healing process was taking place but in the control area (Figs. 21a,b), there was a highly organised advancing bony front with parallel trabeculation from the periphery of the defect towards the centre. The pattern observed on the implanted side was very different. The advancing bony front was less well orientated and lacked the fine trabeculation seen on the opposite side. The presence of implant material appeared to be disrupting the organised pattern of ossification. It might have appeared as though small islands of stained calcified matrix were present within the implant material. They formed no regular pattern and appeared as isolated areas. This could have been interpreted as calcification of areas of the matrix itself. Another, perhaps more credible explanation may be that the soluble component of the implant was lost initially and the space was taken by the ingrowth of osteogenic granulation tissue, which deposited bone. There seems little evidence to support the theory that calcification may be induced by the presence of collagen itself, as the pattern of calcification which might be seen would be expected

to be widespread and dense, not sparse and localised as shown in Figs. 22a & b.

The surgical defect at 28 days was still much in evidence on the implanted side and implant material still present, while the control area was almost fully replaced by bone (Figs. 23 & 24). This finding is verified by Figs. 25 and 26 in which tetracycline bone staining has shown that there was regular organised ossification at the control site and a disordered retarded pattern at the implanted site. Collagen material was again in evidence and all the bone staining appeared around the matrix remnants, none appearing within.

The control area was seen histologically to be completely replaced by bone at five weeks (Fig. 28) and employing bone staining (Fig. 30) the organised pattern was evident. Figure 30 showing the advancing bony front is stained only with tetracycline, as after week 2, the haematoporphyrin was discontinued because of the toxic effect seen in animal A14. By comparison the implanted area is beginning to conform to a trabeculated pattern in parts (Fig. 29a). This shows staining with both tetracycline and haematoporphyrin (given at week 2) and Fig. 29b shows one of many isolated foci of ossification. At 42 days the histology and bone staining was little advanced from the appearance at 35 days and only at 84 days did the implanted area show good bony trabeculation. This seems clear evidence that although the implant material did not excite a foreign body reaction, it had to be eliminated before ingrowth of osteogenic granulation tissue could take place to allow the area to heal by the deposition of bone.

The undecalcified sections in this investigation have clearly shown that the advancing front of osteoblasts was regularly arranged on the control side and advanced from the periphery to the centre of the defect. The implanted area demonstrated that this orientation was completely lost and there was a lack of the fine trabeculation seen at the control site. Fundamentally the same repair process was taking place; the lacunae on both sides showed a similar incremental pattern. More localised areas of ossification were noted in the implanted area and in general the ossification appeared to show a more haphazard picture. Such a disorientated picture did not conform with the pattern of calcification seen when collagen was transplanted into the rabbit peritoneal cavity (Mergenhagen et al 1960). An ordered pattern of calcification of collagen was said to be seen at this location. The studies by Devore and others failed to comment on the pattern of calcification seen with collagen implants. Devore's study was also limited by the lack of observation between two and eight weeks.

In this investigation, it would seem possible to offer an explanation of the way collagen is treated by the tissues. The body recognises the need to deal with any space occupying material or foreign protein. Materials which are not biocompatible may cause an intense inflammatory reaction with the production of granulomas and the appearance of multinucleate giant cells. The area may then settle to produce a more quiescent zone of chronic inflammation. Materials designated as biocompatible, such as metals and silicones, eventually may become enveloped by fibrous tissue containing giant cells which help to isolate the implant. Biodegradable materials may cause the tissues to respond in a

different way and if the necessary enzymes and biochemical processes are present locally, the implant will be lysed. It is known that haematoma and liquified fat will delay wound healing until they are disposed off, when healing will progress normally. In this investigation the acute inflammatory response seen histologically can only be described as comparable with a normal response to injury. In all the sections examined, there was a marked absence of giant cells in relation to the collagen implant. It can be said that the material was therefore not subject to a fibrous reaction but that local tissue collagenases were responsible for its dissolution and it had been dealt with in a manner comparable to other biological "space fillers".

Collagenases have been found in a number of mammalian tissues and are produced by a variety of cells (Chapter 1). Their mode of action appears to be to cleave the triple helix. It would follow therefore that the greater the cross linking, the longer the time for degradation and this has been shown experimentally (Devore 1977). Tissue fibroblasts activated by macrophages are thought to be the effector cells of tissue destruction by collagenase (Laub et al 1982). The cell population is prominently fibroblastic in the healing wound so it is assumed that as well as producing tropocollagen molecules, they are also digesting the heterologous matrix.

The use of a mixture of soluble and insoluble collagens has presented the local cell population with an easily digested soluble matrix interspersed with a more highly cross linked component which is more difficult to digest. The implant matrix in evidence in Figs. 13, 15, 17 and 19 probably mainly consists of the insoluble

component which retards the progress of osteogenic granulation tissue, does not directly calcify and must therefore be removed before the wound can heal.

It may be assumed that however the repair tissue is guided into the defect, it can easily replace blood clot which is probably lysed just in front of the advancing granulation tissue. Soluble collagen can similarly be easily digested by collagenase and may not significantly retard the progress of healing. The presence of insoluble collagen may be the component which causes the healing process to be delayed and it must be eliminated before the area can be replaced by bone. Implant material was detected up to five weeks after surgery in this study and this is longer than in the other studies (Colago et al 1965) but compared favourably with the time taken for the material in Devore's study (1977). This resulted in healing taking nearly twice the time taken by the control area.

In order to further assess the reports of authors that collagen could enhance wound healing, it was hoped that the detection of the early microcirculation up to a period of three weeks, in parallel with the histological evidence, may have proved conclusive. Unfortunately, radiographs of the operated areas after infusion of Hypaque failed to demonstrate an increase in opacity and therefore did not show the degree of neovascularisation in the repair tissue. Thus it may be assumed that the radiological technique, the infusion technique, the biological model or a combination of these factors was at fault.

The infusion technique may be questioned and several reasons for poor contrast filling have been suggested by Rubin (1964), Rubin et al (1964) and Skoglund et al (1978). The reasons suggested

include low infusion pressure; too large a particle size of the medium; vascular spasm as a reaction to the dye and a reaction with platelets or other cells in the circulating blood to form micro-precipitates, which are too large for infusion into bone capillaries. It was also considered by Carlsson et al (1960) and Lundskog et al (1968) that micropaque (particle size $<0.5\mu$) in 50% or 25% dilution was insufficient for complete filling of the vascular bed. The particle size of Hypaque™ is also less than 0.5μ and the infusion pressure used in this study was similar to that used in other investigations. The administration of heparin prior to the dye infusion should have minimised cellular aggregation of blood components and thereby prevented the formation of micro-precipitates. However, only a 25% dilution of the dye was used and vascular spasm possibly as a result of a cooling infusion may have caused poor dye penetration. Although initially the infusion was given at 37°C , its temperature was not kept constant over the whole of the procedure and it was probably at room temperature after death of the animal. According to Albrektsson (1981), the observable contrast medium circulation in bone stopped with death of the animal or very shortly afterwards, thus there may have been insufficient time for the infusion of contrast medium before death in this study.

Theoretically Hypaque™, with a particle size of less than 0.5μ , will readily fill any tube which has the diameter of a blood capillary but it is questionable whether this is a satisfactory comparison with vessels in situ in a biological system. Albrektsson (1981) has also shown that micropaque and Indian Ink contrast media, on average, will fill less than half of the functioning vessels in

a defined bone compartment in the rabbit tibial metaphysis. The results of his study further showed that the range of functioning vessels filled with micropaque was from zero to 81%. This incomplete filling may have resulted from blockage by contrast medium or may represent a functional resting state in the bony vascular bed as described by Rhinelander et al (1968). Anatomical factors may also be significant; according to Debruyne et al (1970), using the guinea pig model, the nutrient artery branches may enter the marrow spaces at an acute angle and they are often extremely narrowed (2μ to 4μ) thus not allowing sufficient filling with contrast material.

Although the method used would appear to be satisfactory, in practice a more sophisticated approach to the neovascularisation of the areas in this study would have been appropriate. Such a study would have to be prepared in a similar manner to that described by Albrektsson (1978), perhaps using an implanted titanium bone chamber. Such an involved study could only be undertaken as an entity in its own right.

Finally, the radiological technique may have been inappropriate. The quality of the film used and the apparatus may not have been able to achieve the high degree of resolution required.

The results from this research will now be considered in more detail with the comparable study by Devore (1977). The histological evidence from the animal studies should be directly comparable. In Devore's study a large number of animals were used and collagens with different cross linkages were compared. The impression of improved bone healing was shown by the formaldehyde

cross linked collagen which was present for up to 42 days at the implant sites. At five weeks, it was reported, new bone was seen forming at the graft site with a distinctly stellate pattern and the graft material was virtually resorbed. At one year, the area was shown to be fully healed. No mention was made of the histological appearance of control areas, we are only informed that they healed.

In this thesis the test material was eliminated by five weeks and at this time, the process of repair was delayed and there was no pattern to the irregular ossification in relation to the graft material. At three months, in this research, control and implanted areas were healed. Contour of the control area had been better restored. Why do the results of similar experiments appear contradictory? The article by Devore (1977) was not reported in depth, specific times were chosen for the results and control areas were omitted. No figures were given concerning the numbers of animals displaying the improved healing or the specific pattern of calcification mentioned. It is clear in this thesis that the presence of a similar collagen (comparable by the similar periods taken for elimination from the test site) retarded progress and delayed bony repair. If a few specific times were taken out of context in this thesis, then the reported results could have been different and probably more supportive. This could have been further enhanced by omitting the findings in the control areas. One can only speculate about dissimilar results from comparable research, perhaps unsupporting results have been excluded or only specific examples used, with the intent of clarifying the data but with the result of unwittingly altering the conclusions.

An alternative method used to further evaluate and investigate the effect of the collagen implant on the host was to look specifically at the immunological response to the material.

The immunological response to implanted collagen did not conform to the primary response to a foreign antigen. A slow rise in antibody level would be expected after an initial delay period of seven to 10 days then a more rapid rise in detectable antibody over five to seven days, followed by a gradual fall to a basal level over the next three weeks. If the animal has already been exposed to an antigen and subsequently re-exposed, then in the first seven to 10 days there is no delay period but a rapid rise in antibody levels to a far greater extent than the primary response. This elevated level of antibody persists for a longer period before slowly declining. This response is termed a secondary response.

It is evident that the response shown by Fig. 33b does not appear to be a classical primary or secondary response curve; it seems to combine the high rise in antibody level within seven days of a secondary response, then shows a gradual primary response decline. It is of interest to speculate why this should be so, especially in the light of the inconclusive evidence so far reviewed in the literature cited in Chapter 1.

The majority of reports showing positive antisera to collagen have resulted from hyperimmunizing animals with collagen preparations in Freund's complete adjuvant with repeated applications subcutaneously or intramuscularly (Kirrairie & Glynn 1968; Steffen et al 1970; Schmitt et al 1964). Collagen injected intravenously failed to produce a detectable antibody response (Rubin et al 1968).

Devore et al (1974) further examined the antibody response to repeated injections of a soluble collagen preparation in Freund's complete adjuvant and also investigated the antibody response shown to collagen implanted in bone. Antibody responses were shown by a gel diffusion technique. The work showed that eight days after a third injection of antigen, a single precipitin line response was exhibited by the technique, indicating one dominant antigen in the collagen-adjuvant material. By comparison, and with the same method, no response could be demonstrated in any of the 12 animals used when the collagen was implanted into bone. Serum from these animals was examined one week and four weeks postimplantation. Such a lack of response has not been verified by this study. Figs. 33a & b clearly demonstrate that detectable antibodies are produced to collagen implanted into bone. The results shown by Devore et al (1974) compare favourably with other similar studies using injected soluble collagen in Freund's adjuvant, one diffusion line being indicative of a dominant antigen. The failure to show an immune response to collagen implanted into bone was questioned and several explanations were offered by the authors. They suggested that the cross linked collagen did not behave as an immunogen. This suggestion is not substantiated by the results in this thesis.

To explain the antibody curve and the differing opinions, the present knowledge of the immune system concerning "self" recognition may be relevant.

In early lymphocyte development those T and B cells with receptors for antigenic determinants on "self" proteins are eliminated or suppressed so that the immune system responds only to foreign antigens. Part of the response to any foreign protein is

played by the memory T cell such that a secondary response to the same antigen is greater and faster (Triplett 1962). The host's immune system is genetically capable of responding also to "self" proteins but learns not to do so. If tolerance to self antigens is compromised, then T or B cells or both may be primed to react against host tissue and produce a so-called autoimmune reaction. Regulator T cells interact with B cells and other T cells by recognising foreign antigen attached to the surface of an interacting cell. These regulator T cells may communicate with target lymphocytes by secreting helper and suppressor factors (Tada & Okumura 1979). Thus when T cells are stimulated by antigen, they are seen to divide and differentiate into activated effector cells and cytotoxic T cells which are able to dispose of foreign antigens. Helper T cells and specific T and B lymphocytes respond to the presence of antigen and also secrete lymphokines such as macrophage migration inhibition factor (M.I.F.) to activate these cells and increase their efficiency at phagocytosis. Lymphocytes also secrete interleukins such as interleukin 2 (T cell growth factor) which stimulates activated T cells to divide and proliferate. The suppressor T cells depress the response of the T and B cells to antigens. It is seen then that helper T cells and suppressor cells act as synergistic regulators of the immune system (Amos 1981).

All mammalian collagens, as discussed in Chapter 1, are structurally very similar, and in the immunologically mature animal, the normal response to a "self" protein is suppressed. If the system is then exposed to a high level of antigen which is structurally very similar to self then the memory T cell may or

may not recognise it as foreign. If it is recognised as "self", then no antibody response may be initiated and this may explain the lack of positive findings in some reports, which may perhaps show species specificity.

If the material is initially recognised as foreign and memory T cells are primed, then the immunological balance may be in favour of the production of helper T cells which will specifically activate B cells and quickly produce high levels of antibody, as shown by Fig. 33b. However, as the heterologous collagen molecule resembles the host natural collagen and the immune system is primed to suppress self antigens, then it would be expected that the initial response by the T helper cells would change rapidly to produce an increasing level of suppressor T cells. The immunological balance then tips in favour of suppression of the antibody response and the antibody level is seen to diminish as the level of suppressor T cells rises. This may also parallel the dissolution of the implant material. In this study by week 9, after implantation, the antibody response shown had fallen to a basal level and the immunological balance was then in favour of T cell suppression.

Clearly if this hypothesis is credible, then there could also be species specificity and individual variations which could explain the range of reactions seen in animal models and in the human reactions to a test dose of Zyderm™.

In neither the animal study nor the human implantation study in this thesis, were any adverse effects clinically evident that could be attributed to the implant material. The ability of T cells to activate macrophages is an important step in cell mediated immunity. Antigen triggered the secretion of lymphokines by memory

T cells, such as macrophage migration inhibition factor, mitogenic factor, chemotactic factor or skin reactive factor. Histologically, after the early acute inflammatory response with polymorph infiltration, the area is filled by an accumulation of macrophages, lymphocytes, mast cells, epithelioid and giant cells often with tissue necrosis and the formation of granulomata.

There was no evidence of granulomata or tissue necrosis seen in the animal or human studies. The animal study showed histologically only an acute inflammatory reaction which did not last longer at the implanted site; both control and implant areas showed that inflammatory cells were gone by 10 days. There was neither a dense macrophage infiltration seen nor evidence of foreign body giant cells related to the collagen. A cell mediated response of a delayed hypersensitivity type was not demonstrated and the response to a heterologous collagen implant in the rabbit was predominantly humoral.

The major antigenic determinants of bovine collagen type I are said to be located at the non-helical terminal ends of the molecule (Rauterberg et al 1972) with only about 20% of the antigenic determinants related to the helical portion (Timpl et al 1971). Pepsin digestion will remove the major antigenic determinants. The presentation of pepsin digested collagen to the rabbit, as in this thesis, induces an immediate antibody response. This is quickly followed by antibody suppression.

If a similar antibody response to that shown in the animal model is seen in the human situation, then a temporary rise in circulating antibody level should not produce any clinical symptoms.

Radiologically the results of the animal work compared favourably with those seen in the histological study. The human study was assessed radiographically so that a comparison could be made with the animal radiological findings and so infer the underlying pattern of healing seen with the collagen implant material.

Animal experiments which are properly standardised and use identical controls should produce meaningful and reproducible results. The major disadvantage of any animal work is that there can always be species specificity and direct comparisons with the human situation cannot be made, only inferred (Autian 1970, 1981). This must therefore be considered when the results are interpreted.

The results from the histological study of rabbit mandibles, using decalcified and undecalcified sections, showed that the collagen matrix itself did not calcify. Figure 43 (pig mandible) showed that there is little change in the radiodensity of the areas when a collagen implant is placed in situ. Any change in radiodensity must therefore have been caused by the deposition of bone.

The earliest radiographic changes were seen 10 days post-operatively. On the control side (Fig. 36 L) the original clear cut surgical margin had become less distinct and this was again shown at 14 days (Fig. 37 L). At 14 days on the control side, the area was beginning to show an increasing opacity. The histological picture at this stage (Fig. 18) confirmed that the osteogenic granulation tissue was laying down bone. At three weeks, the stained undecalcified sections (Figs. 21a,b) showed organised bone trabeculation and this was confirmed on the control side radiographically by a more dense radiopacity. It was also noted

radiographically at this stage that the lower border of the mandible showed evidence of reconstitution.

The implanted areas at 10 days and 14 days comparable to the above showed histologically that healing was occurring but compared with the control areas, it was retarded (Figs. 15 & 17). The original defect was still very obvious radiographically at four weeks on the implanted side (Fig. 39 R) whereas the control site (Fig. 39 L) showed good bone deposition and the lower border of the mandible was reconstituted. These radiographic changes paralleled the bone deposition seen by microscopy (Figs. 24 & 25a,b) on the control side. The less dense radiographic change on the implanted side confirmed the retarded bone deposition seen histologically (Figs. 23&26). The impression up to week 5 was that histologically and radiographically the test site had shown retarded healing.

The radiographic appearance at week 5 did not follow this pattern. The control area (Fig. 40 L) shows bony regeneration around the cut tooth apices but the lower border was not yet reconstituted. Bony regeneration was also seen at the site of the implant but the lower border on this side (Fig. 40 R) had been reconstituted. With regard to the microscopy of these areas (Figs. 27a,b & 28), the implant material was no longer visible and woven bone was seen (Fig. 27a). Histologically the appearance at the control area (Fig. 28) was of more complete bony regeneration. It is important to note that the radiology and histology are of the same animal. Comparing Figs. 29a,b with Fig. 30, the undecalcified sections again exhibited a more organised pattern of bony replacement at the control site but the implanted area did contain much stained bone albeit in a more disorganised fashion. This

would indicate that when the implant material had been disposed of, then the osteogenic repair tissue was able to invade the whole area.

The earlier impression of retarded healing due to collagen is confirmed radiographically at six weeks (Figs. 41 R&L). Both areas showed bony deposition, but the lower border was reconstituted only on the control side.

At 12 weeks both defects were filled with bone and showed comparable radiological densities, but both had notching of the lower border which seemed more pronounced on the implanted side (Fig. 42 R). Devore's similar study (1977) showed that the collagen implanted area developed less notching than the control and the implant area was thus better restored. This is not confirmed by this study. The earliest radiological evidence of calcification with implanted collagen was shown by Fabinger et al (1980) to be at two weeks and this time verifies the radiographic changes at three weeks in this study. Devore's study does not comment on the radiographic appearance of the period between two weeks, when no changes in the radiolucent regions were seen and two months when the defects were largely filled with bone. Devore's study utilised a solid collagen matrix which was soaked in saline prior to implantation. The proportion of collagen in the material was not given. The collagen paste used in this thesis contained only 44% collagen, half as the insoluble material and half soluble. A high percentage of fluid was therefore present which may have been lost immediately after implantation, leaving the area under-restored. This thesis agrees with Devore in that the control site will show notching of the lower border and this may be due to the shrinkage occurring in connective tissue as it matures. The

difference in the implanted group in this thesis compared with Devore's may be that a more dense collagen implant was able to restore tissue contour to a greater extent.

Support for this hypothesis is provided by the use of Zyderm™ for soft tissue recontour. This material contains soluble collagen which constitutes 25-30% of the volume injected only. Over-correction of the defect is advised and a variable number of treatments has to be given before sufficient collagen matrix has been deposited to improve the contour. The soluble portion of the collagen paste described in this thesis may also be eliminated quickly and with loss of the saline and Dextran™ only 22% insoluble collagen may remain within the implanted area. This would appear to be considerably less than the block of insoluble collagen used by Devore (1977).

It may therefore be expected that the implanted area described in this thesis would show a greater degree of notching than the control site and that using this material, total recontour of the lost bony tissue could not be achieved.

The animal work in this study set out to examine some fundamental questions concerning the suitability of a specially formulated collagen paste for use as a bone implant material. The results obtained have been used to consider the findings in previously published works. There is little doubt that the collagen paste is biocompatible and it appears to be resorbed by 28 to 35 days. There is no evidence in this study that collagen induced calcification directly when implanted into bone. The undecalcified preparations clearly showed calcification around the matrix but these areas were localised, sparse and disorganised when compared to

the control areas. The organised pattern shown by Devore et al (1977) has not been demonstrated in this work nor confirmed by any other reports. The number of animals showing organised calcification is not mentioned by Devore et al (1977) and no control areas were shown; thus the value of this work is in question. An uncontrolled study using two dogs by Krekler et al (1980) examined tissue at only two specific points in time, four and five weeks post-implantation. Their conclusion of a direct calcification of the collagen matrix in the periodontal pockets in these two dogs is again limited by the number of animals observed and the period of observation. If control lesions were disregarded in this thesis, a case could be made for collagen calcification but the impression in this research is that healing was retarded by the presence of the implant and mineralisation of the implanted area was delayed. The collagen matrix is shown to act as a barrier to osteogenic granulation tissue and must be removed before the area heals. In considering other authors' results, the number of animals used, the characteristics of the collagen matrix, the times and frequency of observations and the use of controls, must all be considered before any valid conclusion can be drawn.

This study has also demonstrated the immunological effects of bovine collagen in the rabbit. The material acts as an immunogen and levels of antibody were detected by an ELISA. They showed that an initial antibody response is later suppressed and there seemed to be no clinical sequelae to the immune response.

The physical properties of the material were satisfactory and it was easily moulded into the desired shape. There was lack of complete restoration of the bony contour which may have been due to

a loss of initial volume of the implant material after fluid and soluble collagen were resorbed.

Thus the animal work showed, biocompatibility, biodegradability, a slower rate of healing compared to a control, an immune response and lack of full restoration of the bony matrix. The human implantation study will be discussed in the light of these findings.

One hundred consecutive patients who required a bony defect in the maxilla or mandible to be treated were studied clinically and radiologically. It was assumed that changes in radiological density in radiographs of these healing areas would be comparable to those seen in the animal study, and that a similar healing process would be taking place in both studies.

In this human study no adverse reactions which could be attributed to the implanted collagen were shown locally by the appearance of granulomas or other pathological tissue at the site of the implantation. No patient complained of generalised symptoms.

The sample used was, however, small in comparison to the 9000 patients used for the ZydermTM trial by the Collagen Corporation. Their figures showed that 3% of this sample developed a local reaction to the implant material when given a subdermal test aliquot. Using this figure of 3% in the 50 patients who were implanted with collagen only one or two patients would be expected to react; none, however, showed any untoward reaction. This lack of local effect when collagen is placed into bone would require further examination on a much larger study. The progress of the two groups used for the human study has by comparison and

inference from the animal study, shown the effect of the material.

Each of the 100 patients was given a standard post-operative instruction sheet which indicated the minor complications, usually pain and swelling, which might occur immediately post-operatively, the recommended analgesic preparations and instruction on local wound cleanliness. They were asked to return immediately if the pain became persistent, the swelling did not resolve or there was any local tissue damage at the site of operation. In all, 12 patients returned before their first review appointment at two weeks. Only four of the 12 were from the implanted group, of whom three required reassurance only; an antibiotic was prescribed to treat a localised cellulitis in the fourth. In the control group, eight patients returned, four requiring reassurance and an antibiotic was prescribed in four cases, again for persistent local infection.

At two weeks, 89 patients returned for review. Clinically, the areas had healed well, although four patients still complained of some minor discomfort.

There was no evidence in the implanted group that the presence of collagen had caused a severe and extensive acute inflammatory reaction as described by Hunt & Benoit (1976) when evaluating AviteneTM. The clinical impression was that the material was safe and as no adverse reactions were seen, it was not acceptable to the patients to test for antibodies. Venepuncture was discussed with patients but they declined to enter the trial if this was a condition. They did agree that if a local reaction to collagen was evident, then their serum could be examined for anti-collagen antibodies.

Each patient was given a review appointment for three months after their surgery. At this appointment, seven patients in the control group and nine patients in the implant group failed to return (Tables 6.2 and 6.5). Patients who failed to return are not considered in the discussion. Unfortunately this reduced the number in each group which then made statistical data more difficult to interpret. The total figures for failed attendance at six and 12 months were 20% and 28% respectively (Tables 6.3, 6.4, 6.6 & 6.7).

The progress of individual patients is shown in Appendices 1 to 4 and Tables 6.2 to 6.11 are a compilation of this data. A change in radiological density at three months was considered to be indicative of healing within the bony cavity provided the patients were totally symptom free. A similar criterion was used at six months so that the degree of healing could be directly compared with that at three months. At one year it was seen that there could be radiological variation within each group. Complete bony regeneration in areas where both cortical plates had been fenestrated was not usually seen. If collagen had had osteoinductive potential, a difference between control and implanted areas when both cortices were fenestrated would have been evident. The degree of healing shown on radiograph at 12 months was divided into three categories, instead of the two used at the three and six month intervals, in order to demonstrate this point.

In Appendix 2, it will be seen that two female patients, S.R. and N.S., both in the control group, required the operative site to be re-explored due to persisting local infection. Both areas were in the maxilla, one was small and one greater than 2 cm. It is not

uncommon for some apical areas to require re-exploration and this probably results from failure to remove all infected tissue initially. A re-exploration rate of 2% was considered acceptable.

The study served as satisfactory comparison between the implanted and the control groups; the numbers of patients were equal in each group and the number of defects of comparable size in each group was similar. The number of patients absent from review increased with the duration of the study and this made the interpretation of the results more difficult. At three months, in the implanted group, 58% of the original total showed no change in radiological density and 28% showed an increase in opacity. In Appendix 1 it is seen that the majority of the lesions showing this increased opacity were originally <1 cm in diameter. By comparison, 64% of the control group showed evidence of bony healing by three months and only 18% showed no change in radiolucency. By six months, however, comparable figures of 76% in the implant group and 66% of the control showed evidence of bony deposition in both the implanted and control groups.

After one year the figures showed that 44% of the implanted group and 54% of the control group showed the areas to be fully healed by bone deposition. Partial filling of the original area was shown by 28% of the implanted group and 10% of the control with no radiological evidence of repair being demonstrated by only 2% of the patients in the implanted group and 6% in the control.

Tables 6.8 to 6.11 compare control and implanted groups. At three months similar numbers of patients returned in each group, 41 in the control group and 43 in the implanted group. If the healing of lesions in each group is assessed, dividing them into small,

medium and large categories, then statistically there is no difference in healing with respect to the size of the original bony defect. In the control group, $P = 0.2259$ and in the implanted group, $P = 0.4591$. This would indicate that the size of the lesion treated had no effect on the progress of healing taking place. In the control group, three of the six figures used in the statistical analysis were less than 5 in total, and in the implanted group, two figures are below 5; statistical interpretation of such low numbers may be misleading.

Bone deposition in control and implant groups at 3 months is compared in Table 6.11a; 32 patients show bone deposition in the control groups and 14 in the implant group. No radiological change was seen in nine of the control group and 29 of the implant group. If these figures are directly compared, then a significant difference is seen between these groups ($P = 0.0001$). The radiological evidence at three months in the human study thus favours the opinion that healing is retarded in the implant group by comparison with the control group. This view directly parallels the animal work showing that until the implant material is resorbed, healing is retarded. There is a difference in the time scale between the animal model and man; traces of the implant material in the rabbit are present until between four and five weeks, and the implanted area is healed by three months, shown radiologically and microscopically. This is not so in the human situation and most implanted areas are still radiolucent at three months, becoming radiopaque by six months.

The radiological evidence at six months compares similar numbers of patients, 39 and 41 (Fig. 6.9) and it would appear that

the size of the lesion has little effect upon healing. It is again seen that many of the numbers used in these tables are less than 5, and therefore for statistical purposes they are unsatisfactory. Comparing healing between control and implant groups at six months (Table 6.11b) there is no significant difference ($P = 0.2717$) and the majority of patients in both groups demonstrate evidence of healing by this time.

Tables 6.10 a&b comparing healing at one year with respect to the original size of the defect, have too many values below 5 for statistical evaluation. Figures for control and implant groups at this time show that similar numbers of patients returned for review, 70% and 74%. In the implant group, 28% showed partial bony replacement and 44% full bony healing. The comparable figures in the control group were 10% and 54%. No radiological evidence of bone deposition was found in three control and one implanted patient. Comparing these figures, there would appear to be no difference ($P = 0.0572$). Unfortunately, using this data, an osteoinductive role for collagen cannot be supported or refuted.

The early phase of bone healing seen radiographically in the human implantation study was considerably delayed when compared to the control sites. This is comparable with the findings in the animal study which showed a similar delay radiographically in the collagen treated areas. This finding parallels the slower replacement by osteogenic granulation tissue seen microscopically at the implanted areas. The inference from this work is that the delay in healing seen in the human study was caused by the presence of the material. If the healing seen radiographically is considered at one year, then 44% of the implanted group showed the area to be

totally replaced by bone and 28% showed only partial replacement. This may reflect a delay in bony replacement or that the healing process had stopped at a specific time and the lucent area was replaced by fibrous tissue only. All patients were symptom free.

When these findings are considered, it is difficult to accept the very positive report of the similar implantation study and Zetzmann et al (1982). A success rate of 92.5% in cases of third molar removal, cyst implantation and partial mandibular resection was reported. These cases were treated with a new collagen material and the results were assessed radiographically at 14 months. In translation there are linguistic difficulties, but the report does not comment on the variations seen in healing shown by this research. With equal confidence, the author's study could report a similar figure. At review after one year, 74% of patients with the collagen implant in this research, returned and 72% showed bony healing - a success rate of 97%. The interpretation of any results must therefore be circumspect.

Cooperman & Michaeli (1984²) have shown in a prospective human implantation study that two patients (3%) developed a local reaction to subdermally injected Zyderm™. One of these areas was biopsied and showed a mixed inflammatory infiltrate around foci of the implanted material. They stated that the response was self-limiting in the other patient. Their study also found increased antibody levels, measured by radioimmunoassay to injected collagen in one patient at six months and the other at one year.

An interpretation of these findings may be made with reference to the animal work of this thesis. A hypothesis has been made that T

cell suppression becomes dominant after an initial helper T cell response to implanted collagen. Antibody levels after a rapid initial rise will quickly fall. Soft tissue recontour is achieved by repeated injections of Zyderm™ and with individual variation in the immune response, suppression of a helper T cell response may be delayed. Antibody levels after repeated injections may therefore increase and remain at an elevated level.

An alternative explanation may be that denatured collagen, in the form of gelatin, which is used extensively in food preparation, may cause susceptible individuals to develop and maintain antibodies to parts of the collagen molecule (Swarbuck et al 1979). No pre-exposure level of antibody was obtained in either of the subjects in Cooperman & Michaeli's report.

The materials used in all the studies discussed was type I collagen and if there is a connection between auto-immune disease and collagen antibodies, then so far type I has not been implicated. Reaction to type II collagen only has been reported in the experimental animal work on arthritis (Trentham et al 1978; Cremer et al 1983).

In the present state of knowledge, it would still be unwise to continue with collagen implantation in patients who have shown an adverse clinical response.

A study of the immunology of collagen in man with direct measurement of the specific T cell response should clarify the situation further.

This thesis was designed to investigate the properties of a specially formulated heterologous collagen paste both in vivo and in vitro and then to use this information to predict and interpret its

behaviour when used in the human subject.

It has shown there is no cytopathic effect on human fibroblast tissue culture and no adverse effects seen histologically when placed into a prepared bony cavity in the rabbit. Elimination of the material was considered to be by local collagenase digestion probably by the local fibroblast and macrophage population. The material fulfilled the criteria set for biocompatibility and biodegradability. The material was specially formulated to enhance wound healing and clearly this has not been demonstrated. It has been shown that the material delayed wound healing by preventing the ingrowth of osteogenic granulation tissue. Repair of bony lesions in the experimental animal and in man, shown histologically in the rabbit and radiologically in animal and man, have shown that healing is retarded.

In the experimental animal, the material was shown to induce an antibody response but as the material is structurally similar to host collagen, the response appears to have been suppressed after an initial response.

Direct calcification of collagen was not shown and the material did not appear to have an osteoinductive capacity. It was shown to be safe for human use but offered no advantage, such as accelerated healing, with its use.

It is disappointing to show that a material which theoretically had such a useful potential has, as most of the other materials investigated for this purpose, not totally fulfilled the criteria set. A suitable material to replace areas of lost bone has yet to be found. There are now reports of the use of a combination of collagen with hydroxyapatite as a more suitable material. Several

authors (Gross et al 1980; Hayashi et al 1982; Mettelmeier & Katthagen 1983) suggest that hydroxyapatite may have osteoinductive properties which have been found lacking in the collagen material of this research. They also suggest that this osteoinductive capacity may produce an early increase in strength in bone healing. If this can be substantiated, then the collagen paste used in this thesis, which is shown to be biocompatible and biodegradable, may provide a suitable vehicle for the hydroxyapatite.

In conclusion, the work in this research has provided answers to some of the questions concerning the suitability of collagen as a bioimplant material for bone. Much work is still required to produce an ideal allograft material, but it is hoped the data presented will be useful as a guide in understanding the effects of other biological materials on bone healing.

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APPENDIX 1

RADIOGRAPHIC ASSESSMENT OF PATIENT PROGRESS

CONTROL GROUP (MALES)

Patient	Site	Size	3 months	6 months	12 months
M.B.	Mand.	M	Margins less distinct increased opacity.	Reduction in radiolucent area. Bone of replacement in lower half of lucent area.	Almost complete bony replacement.
A.B.	Max.	S	Increased opacity. Peripheral early trabeculation.	Bony replacement in outer half of original area.	Complete bony replacement.
C.C.	Mand.	M	Increased opacity in bony cavity.	Bony replacement in periphery of original area.	Complete bony replacement.
J.D.	Max.	S	Increased opacification in bony cavity.	Bony replacement in outer half of original lucent area.	Bone replacing three quarters of original lucent area.
S.F.	Max.	M	Increasing opacification in bony cavity.	Reduction of lucent area and replacement with bone.	D.N.R.
K.F.	Max.	S	No change in lucent area.	No increase in opacification of lucent area.	D.N.R.
C.F.	Max.	S	D.N.R.	D.N.R.	No change in lucent area - no symptoms.
I.G.	Mand.	S	No change in lucent area.	Increasing opacification in bony cavity.	D.N.R.
G.H.	Max.	M	No change in lucent area.	Margins less distinct increased opacification.	Bone of replacement in peripheral half of original area.
R.L.	Max.	M	Lucent area replaced by mottled opacity.	Bony replacement at periphery of lucent area.	D.N.R.
J.M.	Mand.	L	Increasing opacity early peripheral bony replacement.	Whole area now replaced with bony trabeculation.	Complete bony infilling.
P.M.	Max.	S	Increase opacity in bony cavity.	Bony trabeculation in periphery of bony cavity.	Complete bony replacement in cavity.

Patient	Site	Size	3 months	6 months	12 months
G.M.	Max.	M	Increased opacity in bony cavity.	D.N.R.	Complete bony replacement in cavity.
G.N.	Max.	S	Early peripheral bony replacement.	Died.	
G.O.	Max.	S	D.N.R.	D.N.R.	Area reinfected.
G.R.	Max.	S	Increased opacity early bony replacement peripherally.	Whole of cavity replaced by bony trabeculation.	D.N.R.
A.R.	Max.	S	D.N.R.	Original lucent area reduced in size but central area still radiolucent.	D.N.R.
S.R.	Max.	M	Lucent area replaced at periphery by opacity.	Lucent area now replaced by radiating trabeculated bone.	Bony replacement in all but small central zone.
P.T.	Mand.	S	No change in lucent area.	D.N.R.	D.N.R.
R.T.	Max.	S	D.N.R.	Bony trabeculation in peripheral half of bony cavity.	D.N.R.
B.T.	Max.	S	Increasing opacification in bony cavity.	D.N.R.	Complete bony replacement of lucent area.
R.W.	Max.	S	D.N.R.	Lucent area replaced by central opacity and peripheral trabeculation.	Complete bony replacement.

APPENDIX 2
RADIOGRAPHIC ASSESSMENT OF PATIENT PROGRESS
CONTROL GROUP (FEMALES)

Patient	Site	Size	3 months	6 months	12 months
A.A.	Max.	S	Increasing opacity over whole defect.	Peripheral bony replacement with central lucency.	Complete bony replacement with central lucency.
A.A.	Mand.	S	D.N.R.	Whole area of operation shows increased opacity.	Complete bony healing.
C.B.	Max.	M	Minimal increase in radio-opacity.	D.N.R.	Complete bony replacement
P.B.	Max.	L	Whole area shows no change in lucency.	Peripherally some increase in opacity of area margin indistinct.	Half of original area shows trabeculated bone. Central area still lucent.
P.C.	Max.	M	Reduction in size of lucent area.	Whole area shows good opacification with peripheral trabeculation.	Whole area filled with trabeculated bone.
C.C.	Max.	S	Increased opacity of whole defect.	Early bone replacement with radiating trabeculae.	Whole area completely replaced by bone.
T.C.	Mand.	L	Area filled with amorphous opacities.	Whole area completely opacified with trabeculated bone in periphery.	Bone in whole of defect except adjacent to teeth.
N.C.	Mand.	S	Margins indistinct loss of lucency.	D.N.R.	D.N.R.
K.C.	Max.	S	No change in lucency.	Area required re-exploration.	Whole area shows increased opacity.
J.D.	Max.	M	Increased opacity at operation site.	Margins indistinct peripherally replaced with trabeculated bone.	Whole area shows bony replacement.
P.D.	Mand.	M	Area shows increased opacity with indistinct margin.	Peripheral trabeculated bone of replacement.	Area healed with coarsely trabeculated bone of repair.
M.E.	Max.	M	Decreased opacity at periphery clearly demarcated from central lucent area.	Small central lucent area still persists. Area replaced peripherally by trabeculated bone.	Small central lucency otherwise completely replaced by bone.

Patient	Site	Size	3 months	6 months	12 months
E.G.	Max.	M	Margin of peripheral opacification only.	Area almost completely replaced by trabeculated bone.	Area completely replaced by normal bone.
D.G.	Mand.	M	D.N.R.	D.N.R.	Original area replaced by bone except for small lucency at root apex.
I.H.	Max.	S	Peripheral opacification of lucent area.	Area now completely replaced by radiating trabeculated bone.	Area replaced by normal bone.
E.L.	Max.	S	Whole area shows increased opacification.	Peripheral radiating bone trabeculation.	D.N.R.
E.McG.	Max.	S	Whole area shows increased opacification.	Area fully replaced by bone.	D.N.R.
L.M.	Max.	M	Replacement of lucent area by peripheral opacification.	Peripherally area replaced by bone. A small central area shows increased lucency.	Whole area replaced by bone except small lucent area adjacent to root apex.
M.P.	Max.	S	D.N.R.	D.N.R.	D.N.R.
M.P.	Max.	S	Increased opacity with a mottled appearance.	Trabeculated bone at periphery of original area.	Area replaced by bone except small lucent area at apex of root.
J.R.	Max.	S	Radiolucent area replaced peripherally by opacified zone.	D.N.R.	D.N.R.
S.R.	Max.	S	No change in radiolucent area.	No change in lucency.	Area re-explored due to recurrent symptoms
A.R.	Max.	S	Radiolucent area replaced by opacified bone at periphery.	Bony replacement at peripheral half of original lesion.	Area replaced by bone except for residual lucency at root apex.
N.S.	Max.	L	Area replaced by opacity showing a mottled appearance.	No change from 3 months.	Area re-explored and infected granulation tissue removed.
K.V.	Mand.	M	No change in lucent area.	Area re-explored.	At 18 months bony replacement in original lucent area.

Patient	Site	Size	3 months	6 months	12 months
J.W.	Max.	M	Lucent area replaced by mottled opacification.	Periphery of area replaced with trabeculated bone.	Bony replacement of lucent area except around root apex.
J.W.	Mand.	M	D.N.R.	Central lucent area surrounded by radiating trabeculated bone.	Bony replacement of area except around root apices.
E.W.	Max.	M	Whole area shows increased opacification.	Trabeculated bony replacement of area.	Bone of a more mature appearance now demonstrated.

APPENDIX 3

RADIOGRAPHIC ASSESSMENT OF PATIENT PROGRESS

COLLAGEN IMPLANT GROUP (MALES)

Patient	Site	Size	3 months	6 months	12 months
A.B.	Max.	S	D.N.R.	Margins indistinct. Opacity increased.	D.N.R.
D.B.	Mand.	M	No change in lucent area.	Reduction in size of lucent area with indistinct margin.	Whole area shows opacification with peripheral trabeculated bone.
J.B.	Max.	S	No change in lucency but periphery less well defined.	Area now shows increased opacity. No new bone seen.	Margins indistinct. Trabeculated bone at periphery of original area.
M.B.	Max.	S	D.N.R.	Margins indistinct. Area shows increased opacification.	D.N.R.
J.C.	Max.	S	No change in lucency of original area.	Opacification of area with coarse trabeculation at periphery.	Area replaced by normal bone.
C.D.	Max.	S	No radiological changes.	D.N.R.	D.N.R.
A.G.	Max.	S	No radiological changes.	Area opacified. Margins indistinct.	Periphery of area shows replacement with trabeculated bone, central area opacity.
A.G.	Mand.	L	D.N.R.	Trabeculated bone replacing whole of original defect.	Bony replacement with some areas of sclerosis.
R.H.	Max.	S	No radiological change.	Margins indistinct. Increased opacity.	Peripherally replacement by trabeculated bone.
W.McC.	Max.	S	No radiological changes.	Increased opacity in whole area.	Opacity shows peripheral bony replacement.
J.McD.	Mand.	L	No radiological changes.	D.N.R.	Increased opacity with marked reduction of radiolucent area with deposition of bone of repair.

Patient	Site	Size	3 months	6 months	12 months
T.McK.	Max.	S	No change in radiolucency.	Area now shows increased opacity.	D.N.R.
F.M.	Max.	M	D.N.R.	Trabeculated bone in periphery of lesion with central opacity.	D.N.R.
A.S.	Mand.	M	Area shows peripheral opacification but central area still lucent.	D.N.R.	Whole area replaced by bone of repair.
W.S.	Max.	L	Peripheral opacity around central lucency.	Area now shows opacification with indistinct margins.	Periphery shows trabeculated bone of repair. Central area mottled opacity.
G.S.	Max.	M	No change in radiolucent area.	Peripheral opacity with coarse bony trabeculation.	Area replaced with bone of repair with lucent bone around root apices.
J.T.	Mand.	M	D.N.R.	Area shows increased opacity with peripheral bony replacement.	D.N.R.
M.W.	Mand.	M	Area shows increased opacification with indistinct margins.	Area replaced by trabeculated bone at periphery.	Whole area replaced by trabeculated bone.
S.Y.	Max.	M	No change in radiolucent area.	Margins indistinct. Increased opacity at periphery. Central lucent area still present.	D.N.R.

APPENDIX 4

RADIOGRAPHIC ASSESSMENT OF PATIENT PROGRESS

COLLAGEN IMPLANT GROUP (FEMALES)

Patient	Site	Size	3 months	6 months	12 months
C.B.	Max.	M	No change in radiolucent area.	Increased opacity at periphery of lucent area.	D.N.R.
J.B.	Max.	S	Increased opacification of radiolucent area.	Trabeculated bone in peripheral half of original area.	Bony replacement with trabeculated bone of repair.
G.B.	Mand.	M	Radiolucent area shows peripheral opacification.	Increase in density of peripheral opacity but lucent area remains around root apices.	Bone of repair in lesion except around root apices - still shows lucency.
A.B.	Mand.	M	No change in radiolucent area.	Peripherally bone of repair well-defined.	Area healed with trabeculated bone of repair.
C.D.	Mand.	S	No radiological change.	D.N.R.	Complete replacement of lucent area with bone of repair.
M.D.	Max.	S	No radiological change.	Margins indistinct. No bone in area. Central area still radiolucent.	D.N.R.
A.D.	Max.	S	D.N.R.	Increased opacity suggesting bony repair.	Area replaced with trabeculated bone of repair except small lucent area around root apices.
C.F.	Mand.	M	No radiological change in density.	Margins indistinct. Whole area shows increased opacity.	Area replaced by trabeculated bone of repair.
M.F.	Max.	S	No change in radiological density.	Margins indistinct. Minimal change in radiological density.	Peripherally bone of replacement. Central area still lucent.
A.H.	Max.	S	No radiological change in density.	Peripheral bony deposition with lucent area remaining around root apices.	Area replaced by bone of repair except lucent area at root apices.

Patient	Site	Size	3 months	6 months	12 months
H.H.	Max.	L	Increased opacity with early bony trabeculation at periphery.	Marked reduction in size of original area with trabeculated bone of repair.	Area replaced by bone except around root apices - still lucent.
E.H.	Max.	M	No change in radiological density.	Margins indistinct but little change in central lucent area.	Peripherally bone of repair, centrally area shows increased opacity.
M.K.	Max.	S	Increase opacity over whole area.	D.N.R.	Area replaced by bone of repair except small lucent area at root apices.
M.K.	Max.	S	No change in radiological density.	Margins indistinct. Central opacity of area now seen.	Area now replaced with trabeculated bone of repair.
C.L.	Max.	S	No radiological change.	D.N.R.	Area infected - required re-exploration.
A.L.	Max.	M	No radiological change in density.	Margins indistinct. Central opacity of original lucent area.	More dense opacity with peripherally bone of repair noted.
A.L.	Max.	S	No change in radiological density.	D.N.R.	Area replaced by bone except small lucency at root apices.
I.McC.	Max.	M	No change in radiolucency.	Central lucent area with peripheral opacity with early trabeculation.	Increased bony trabeculation but central lucent area remains.
S.McG.	Max.	S	No change in radiolucent area.	Increased opacity in whole of area.	Peripheral replacement with bone of repair.
M.McK.	Max.	L	No radiological change.	Margins indistinct. Minimal increase in radiological density.	Opacity increasing with margin peripherally of bone of repair.
H.M.	Max.	S	Margins indistinct	Area now uniformly radio-opaque with peripherally indistinct margins.	D.N.R.
J.M.	Max.	S	Increased opacity in bony defect.	Peripherally area now shows trabeculated bone of repair.	Area replaced by bone with small residual lucency at root apices.

Patient	Site	Size	3 months	6 months	12 months
H.N.	Max.	S	Increased opacity in bony defect.	D.N.R.	D.N.R.
U.O.	Max.	S	No change in radiological density.	Central lucent area with peripheral opacity.	Central lucent area separated from peripheral area of bone of repair.
J.P.	Max.	M	Mottled opacities throughout lucent area.	Whole area now markedly radio-opaque. Small lucency around root apices.	Trabeculated bone of repair in all but small area at root apices.
I.T.	Max.	M	Increased opacity in whole of original area.	Peripherally bone of repair around central opacity.	Bone replacement around well circumscribed central lucent area.
M.T.	Mand.	M	D.N.R.	Increased opacity in central zone with coarsely trabeculated bone of repair peripherally.	D.N.R.
J.W.	Mand.	S	Margins indistinct. Area radio-opaque.	Peripheral bone of repair around central opacity.	Bone replacement in original area.
M.W.	Max.	S	No change in radiological density.	D.N.R.	D.N.R.
E.W.	Mand.	M	Margins indistinct. Area shows increased opacity.	Trabeculated bone of repair well established.	Bony repair of original area.
L.W.	Max.	S	No change in lucent area.	Peripherally trabeculated bone towards central opacity.	Area healed with trabeculated bone of repair.